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STUDIES ON THE BLOOD SUPPLY OF TUMORS IN MAN. IV. THE INCREASED OXYGEN CONTENT OF VENOUS BLOOD DRAINING NEOPLASMS [1] [2]

HOWARD R. BIERMAN, KEITH H. KELLY,[3] and GRACE SINGER, *Laboratory of Experimental Oncology, National Cancer Institute, National Institutes of Health, Public Health Service, Federal Security Agency* and the *Division of Medicine, University of California School of Medicine, San Francisco, Calif.*

The increased vascularity of many types of neoplasms in man has been demonstrated by various methods that have emphasized mainly the abnormal arterial patterns and increased capillary permeability to fluorescein (*1,2*). More recent studies have illustrated an increased temperature over such lesions than over comparable uninvolved areas and further reflect the increased blood supply of such neoplasms (*3*). Other investigations (*4*) have revealed that the blood flow through liver areas containing metastases is more rapid than through uninvolved areas of the same liver, and this differential in flow can be easily demonstrated by arteriographic methods. Furthermore, there are characteristic abnormalities of the smaller vessels within the tumor areas that are manifested by sinusoidal dilatations and in which the radiopaque material appears to persist longer than in the uninvolved areas (*4*).

Since such vascular abnormalities may result in a decreased vascular resistance, the normal head of pressure to these areas might be increased and may well account for the increased rate of blood flow. By the same token, such increased arterial blood flow should be reflected on the venous side of this disturbed capillary bed. Under the influence of an increased head of pressure, rapid blood flow, and sinusoidal dilatation of the smaller

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vessels it was thought possible that more of the oxygenated blood might pass directly from the smaller arteries and arterioles to the venous side, resulting in a physiologic arteriovenous shunt.

Abnormal arteriovenous communications can often be diagnosed from the oxygen content of the blood of the regional veins (*5*). Some forms of vascular intracranial tumors and arteriovenous aneurysms can be detected by obtaining an elevated oxygen content of venous blood drawn from the jugular bulb and may be localized to one side or the other by comparing with the results obtained from the blood of the contralateral jugular bulb (*6*).

Arteriovenous aneurysms when significantly large will cause many local and systemic effects (*7,8*). Cardiac changes have been noted, and frank congestive failure has been reported with extensive arteriovenous fistulas (*9,10*). In general, however, such extensive shunting of blood would not be expected within tumors; so that the local manifestations of arteriovenous by-passing of blood such as bruit, decreased oscillations of the toes and fingers distal to the lesion, and venous dilatation are not usually seen. However, pulsations of some neoplasms, notably metastases from the Grawitz tumor (adenocarcinoma of the kidney), are considered characteristic.

This report deals with a study of the oxygen content of arterial and venous blood serving accessible neoplastic lesions and compared whenever possible with the identical contralateral vessels of uninvolved areas. In some instances good controls or normal values were not available, and the findings in these cases are included for interest with the best comparison possible.

METHODS

The 12 patients studied had neoplastic lesions situated so that the major vein draining the tumor was accessible. Blood samples were obtained by direct puncture of the vessels under adequate local procaine anesthesia or from catheters inserted into the proper vessels as previously described (*2*). The patients were in resting but not basal conditions, and temperature vasomotor stimuli were avoided. Whenever possible the venous sample was taken first from both the involved and uninvolved areas to avoid reflex vascular stimulation, which might possibly accompany the arterial punctures (*11*). The samples were taken as close to the lesion and simultaneously as possible. The blood was drawn into oiled syringes containing heparin with caution to exclude all contact with air. The samples were sealed in the syringe by an air-tight cap and immediately placed on ice. All determinations for oxygen were undertaken within 30 minutes after collection. The oxygen content of the blood samples was determined spectroscopically (*12*), after calibration with the manometric technic. Saponin was employed as the lysing agent in all determinations.

The diagnosis of the neoplasm was established by adequate biopsy in all cases. Lesions with evidence of necrosis were avoided.

RESULTS

The oxygen content of the venous blood draining directly from the tumor in the 12 patients was found to be higher than the oxygen content of venous blood obtained at another site (table 1). In 2 patients the arteriovenous oxygen difference from the involved site was less than 2 volumes percent, and in the remaining patients it was lower than the arteriovenous difference from comparable uninvolved areas. The venous blood sampled from a site draining, but distant from, the neoplasm showed less evidence of increased oxygenation. In general, the closer to the neoplasm the venous samples were obtained the higher the oxygen content. The oxygen content of the venous blood from cutaneous lesions, which were red and exhibited an increased surface temperature, approached that of the arterial blood more closely than when the lesions were without erythema.

In two instances, arterial and venous blood samples were obtained simultaneously *via* catheters manipulated into the main artery supplying, and the main vein draining, the neoplasm. In one case (Tan), with an extremely large liver due to hepatoma, an arteriogram of the liver showed an increased and abnormal vascularity. The arteriovenous oxygen difference between the hepatic artery and vein was 3.15 volumes percent. The oxygen content of the blood from the hepatic vein was 2 volumes percent more than in the right ventricular blood. In another patient (Goo), the renal vein blood from the kidney with the neoplasm revealed an oxygen content almost equivalent to that of the renal artery on two separate occasions (table 1). In another patient (Mea), the right jugular vein had been sacrificed during a deep neck dissection. The oxygenation of the venous blood draining from the tumor site was not markedly increased. The surgery plus intensive irradiation to the right neck and face probably reduced the vascularity of the tumor, and may explain the findings. In the patient Leo, the venous blood was obtained at operation from a vein draining a large ovarian metastasis from an adenocarcinoma of the breast. The mass contained mostly fragments of tumor within a pool of mucinous material and was remarkable microscopically for its avascularity.

In most instances the oxygen capacities of the venous and arterial blood were reasonably close. Despite the minor discrepancies, the percent oxygen saturation of the venous blood draining the tumor areas was always higher than that of the venous blood from uninvolved areas.

DISCUSSION

The increased oxygenation of venous blood draining tumor areas indicates a rapid blood flow through these neoplasms aided perhaps by some physiologic arteriovenous shunting of oxygen or failure of oxygen utilization by the neoplastic tissues.

The high oxygen content of venous blood can be explained by mechanical or anatomical arteriovenous communication developed during the in-

TABLE 1.—*Blood oxygen determinations on 12 patients with neoplastic diseases*

Name	Diagnosis and tumor studied	Artery	Oxygen capacity	Oxygen content	Vein		Oxygen capacity	Oxygen content	Percent saturation
					Draining tumor	Control			
ALV	Osteogenic sarcoma distal medial head of right femur.	Right femoral	17.2	16.3	Right femoral		16.8	12.2	72.6
						Left femoral	17.4	8.9	51.2
BEA	Malignant melanoma of right leg and thigh.	Left femoral	16.0	14.0	Right femoral		16.1	11.1	68.9
						Left femoral	16.0	9.7	60.6
LAC	Malignant melanoma of right thumb.	Right brachial	16.6	15.3	Right cephalic		17.2	14.2	82.5
						Left cephalic	12.9	9.8	75.9
HAR	Malignant melanoma of left thigh:								
	September 1949	Left femoral	14.8	14.0	Left femoral		14.4	12.0	83.3
		Right brachial	13.9	13.1		Right basilic	14.8	6.4	43.2
	November 1949	Left femoral	13.4	12.6	Left femoral		14.1	12.0	85.1
						Right femoral	12.7	6.6	52.0
						Right basilic	13.9	7.3	52.5
GEO	Multiple myeloma lesion of left femur.	Right brachial	14.6	13.9	Left femoral		14.7	11.3	76.9
						Right basilic	14.6	5.6	38.4
BOR	Bronchiogenic carcinoma, metastasis of right femur.	Right femoral	13.6	12.5	Right femoral		12.7	7.1	55.9
		Left femoral	13.0	11.9		Right basilic	12.6	4.4	34.9
TAN	Hepatoma	Celiac	15.7	15.0	Hepatic		16.3	11.9	73.0
						Right ventricle	16.1	9.7	60.2
GOO	Renal (adenocarcinoma of left kidney).	Renal	11.2	10.3	Left renal		11.1	10.3	92.8
						Pulmonary conus	10.0	6.8	68.0
BIO	Lesion of distal right forearm, metastatic from carcinoma of esophagus.	Right brachial	9.8	8.9	Right cephalic closest to the tumor.		10.0	4.3	43.0
					Right basilic at cubital fossa.		9.5	3.2	33.7
						Left cephalic	9.8	1.5	15.3
MEA	Squamous-cell carcinoma right neck involving skin.				Right jugular		18.8	11.3	60.3
						Right subclavian	18.5	10.4	56.4

LEO	Adenocarcinoma of breast metastatic to ovary.	Ovarian	17.5	17.1	Ovarian		16.4	10.8	65.9
YOU	Melanoma in left inguinal nodes	Femoral	17.5	17.0	Left femoral		18.7	14.1	75.4
						Right femoral	17.5	12.1	69.1
McC	Reticulum-cell sarcoma of both legs.	Right femoral	15.8	15.3	Left femoral		16.2	11.1	68.5
					Right femoral		16.4	11.9	72.6
SER	Carcinoma of stomach	Gastric	18.0	17.3	Gastric		19.3	16.6	86.0
						Basilic [1]	17.1	13.9	80.9
WIM	Hypernephroma, bilateral renal tumors.	Right renal	11.9	9.2	Right renal		11.4	8.5	74.6
		Left renal	11.1	8.4		Left renal	11.2	7.8	69.6

[1] Best control available.

crease in tissue mass of the neoplasm or by some vasomotor influence that maintains a constant state of dilatation of the blood vessels. The latter is unlikely, since it has been shown that vessels within some neoplasms do not respond to the usual vasoconstrictor agents while normal vessels do (*13*). It is also unreasonable to assume that these neoplasms fail to remove any of the oxygen and can exist and flourish under these circumstances although, of course, the ability of neoplastic tissue to undergo anaerobic glycolysis is well known. A more likely explanation may be an extremely rapid blood flow increased out of proportion to the metabolism of the tumor.

In six instances the vein draining the tumor contained blood with three to six volumes percent of oxygen higher than from a comparable vein not involved with tumor. The normal renal arteriovenous oxygen difference is reported to be 1.4 to 2 volumes percent (*14, 15*). In patient Goo, the venous blood contained as much oxygen as the arterial blood drawn at the same time for an arteriovenous difference of zero.

The arteriovenous oxygen content of the liver (hepatic vein) is reported by different investigators to be 10.9 to 4.5 $\pm$ 0.8 (*17, 18*) volumes percent in dog and man (*16*). In patient Tan, the arteriovenous difference was only 3.1 volumes percent. Myers and Hickam (*18*) report the arterial hepatic venous oxygen difference for patients with cardiac failure varied from 5.5 to 12.8 volumes percent with a mean value of 8.4. Patient Tan had massive dependent edema with ascites, and the liver at necropsy weighed 10,000 grams.

It should be emphasized that in those instances where good control samples were not available the data are included primarily for interest and support but are not conclusive in themselves.

CONCLUSIONS

The oxygen content of venous and arterial blood serving neoplasms was compared with blood from comparable, uninvolved areas in 12 patients with metastatic neoplastic lesions.

The oxygen content and saturation of the venous blood draining directly from the neoplasm was higher than the venous oxygen content obtained elsewhere. The arteriovenous oxygen differences obtained from the neoplastic vessels was smaller than from uninvolved areas.

The increased oxygen content of venous blood draining neoplastic lesions indicates considerable arteriovenous shunting or lack of oxygen extraction within some neoplasms. These findings strongly suggest an increased and more rapid blood flow through these tumors than in comparable normal tissue.

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INCIDENCE OF TUMORS IN ISOGENIC STRAINS [1] [2]

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One of the reasons for many advances in oncology during the past quarter century has been the development of uniform strains of experimental animals. It has been found, however, that not all animals in susceptible strains bear tumors. This incomplete penetrance of susceptibility genes has puzzled oncologists for a number of years. In mammals, inbreeding is the best method for obtaining genetic homogeneity, but isogenic (completely homozygous) strains cannot be obtained with certainty in species such as mice because it is not possible to have the numerous chromosomes marked at once, and inversions are not available to prevent crossing over. It is possible to obtain isogenic, tumor-bearing strains of *Drosophila*, however. Isogenic strains therefore have been established and tumor incidence determined in two such stocks, since this has not been reported previously for lines isogenic for all chromosomes.

METHODS

It was thought desirable to select strains with low and high incidence of tumors and with and without a difference in incidence between the sexes. Accordingly, the *tu 36 a* strain with the same low incidence in both sexes and the tu^s stock with a relatively high incidence, greater in males than in females, were utilized. During early larval life the tumors in the *tu 36 a* strain are cellular and appear in the fat body or adjacent to the gut. Sections of tu^s larvae have shown fewer cellular elements, consisting of fusiform cells, and a large amount of pigment in the fat body. The main genes for tumor susceptibility lie on the second chromosome in these stocks (*2*, *4*). Selected strains maintained by sibling matings of individuals bearing tumors were used to obtain isogenic stocks, which were then compared to parallel generations of the corresponding selected stock. One *tu 36 a*, selected line was carried in the incubator at $25 \pm 0.5°$ C. The remaining stocks were bred in an air-conditioned laboratory at 25° C. with a wider range in temperature. The same culture medium with live yeast from a common source growing on the surface was used in all experiments.

The method for constructing isogenic stocks is outlined in text-figure 1,

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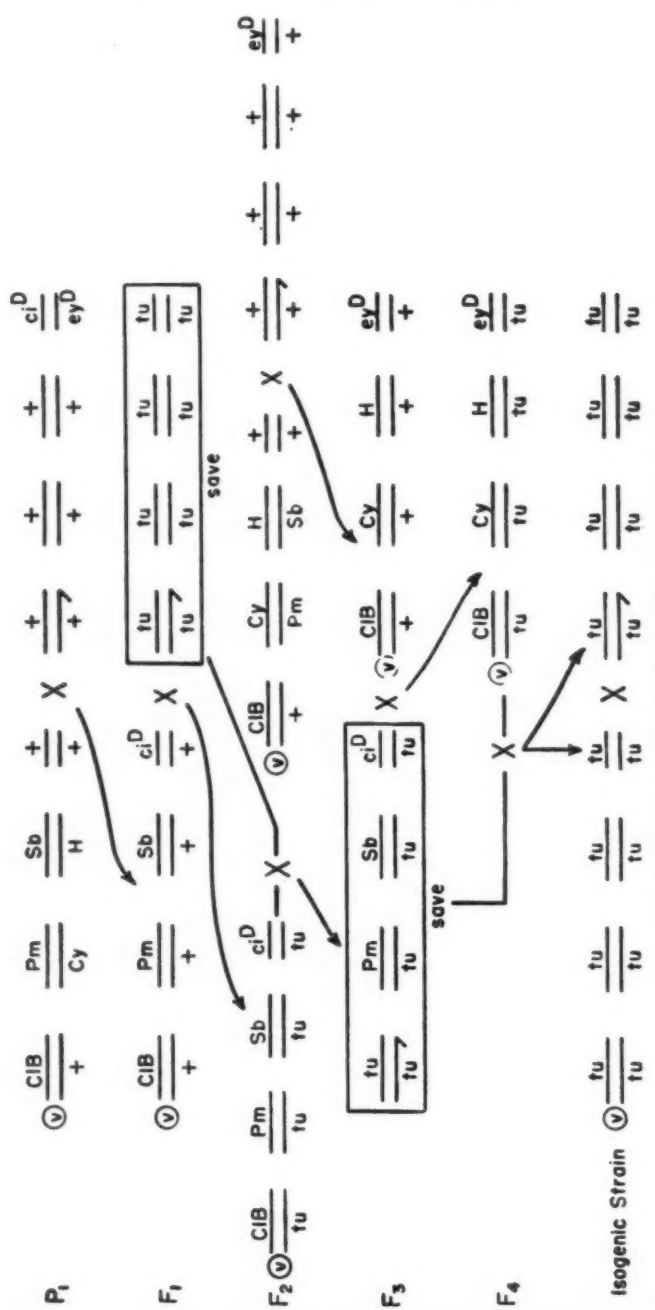


TEXT-FIGURE 1.—Scheme for obtaining isogenic strains.

and is adapted from that described by Bridges (*1*) and Muller (*3*). Inversions were used with dominant markers to prevent crossing over in chromosomes 1, 2, and 3 and dominant markers associated with chromosomal aberrations in chromosome 4. It may be seen in text-figure 1 that individuals heterozygous for all chromosomes from tumor-bearing flies, when crossed to their own offspring in individual matings, will yield a homozygous or isogenic stock that can be distinguished from other siblings in the same culture. The isogenic strains each originating in a single individual mating may then be maintained by individual matings of siblings bearing tumors in subsequent generations.

The details of the method may be followed in text-figure 1. In the P_1 generation two stocks carrying chromosome aberrations to prevent crossing over and marked with dominant genes are crossed so that F_1 virgin females heterozygous for one of these marked chromosomes in each chromosome pair is available for mating to a tumor-bearing male. The male is saved and crossed to a virgin daughter in the F_2, which is heterozygous for *ClB*, *Pm*, *Sb*, and ci^D and therefore bears the chromosomes from the tumor-bearing father as one of each chromosome pair. The male offspring from this cross with the characters *Pm*, *Sb*, and ci^D in the F_3 has the X and Y chromosomes and one of the second, third, and fourth chromosomes descended from the original tumor-bearing male. It is crossed in the F_3 generation to a virgin female heterozygous for chromosome aberrations marked with *B*, *Cy*, *H*, and ey^D. In the F_4 generation virgin females bearing *B*, *Cy*, *H*, ey^D are then mated individually to the male parent which has been saved from the F_3 generation. All unmarked flies in the F_5 generation are isogenic, having obtained all chromosomes from the chromosomes designated as *tu* in the male used in the F_3 and F_4 generations. The offspring from each of these males were maintained separately. It should be noted that the F_3 male has only one of each chromosome from which all the chromosomes of the isogenic strain are derived.

DISCUSSION OF RESULTS

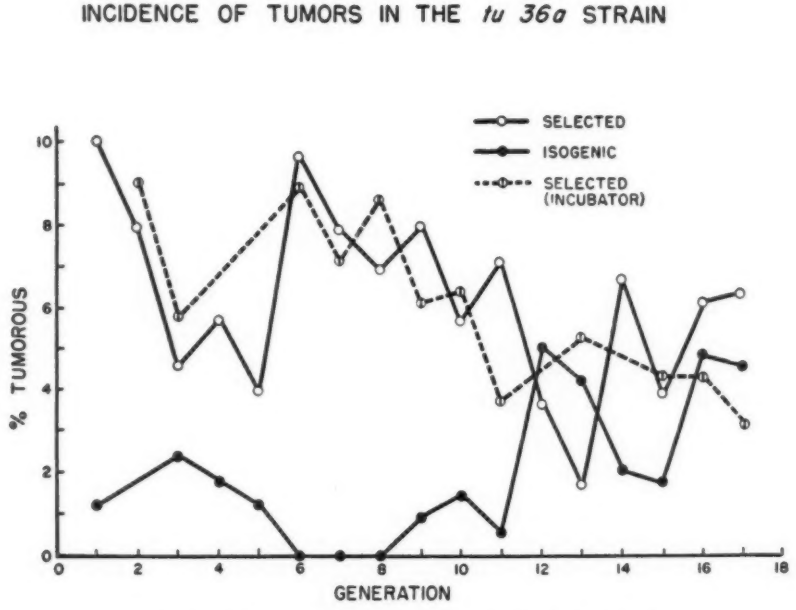
There is a difference between the 2.58 percent incidence of tumors in the isogenic, *tu 36 a* strain and that for each of the selected strains tested ($P<0.0001$ in each instance) (table 1) except for 2.97-percent incidence of tumors in the selected strain previous to isolation of the isogenic strain ($P=0.16$). Tumor incidence was tabulated separately for each sex only in the later generations. During these later generations (table 1) there is no difference in incidence between the sexes in the selected ($P=0.73$), selected at $25\pm0.5°$ C. ($P=0.68$), or isogenic *tu 36 a* stocks ($P=0.97$). There is also no difference between the incidence in cultures of the selected strain raised in the laboratory and in the incubator after the isogenic strain was established (table 1 and text-fig. 2) ($P=0.48$), but in previous generations there is a difference ($P<0.0001$).

It is evident from table 2 that the incidence of tumors in males from the tu^s stock is higher than females in both the selected ($P<0.0001$) and isogenic stocks ($P<0.0001$). The percentage of tumor-bearing individuals

TABLE 1.—*Incidence of tumors in tu 36 a strain*

Sex	Group	Number of generations	Tumor-bearing	Total	Percent tumor-bearing
Male	Selected	9	202	3, 700	5. 46
"	Selected (Incubator)	7	53	1, 223	4. 33
"	Isogenic	9	76	2, 483	3. 06
Female	Selected	9	178	3, 379	5. 27
"	Selected (Incubator)	7	51	1, 087	4. 69
"	Isogenic	9	73	2, 434	2. 99
Both	Selected [1]	14	225	7, 576	2. 97
"	Selected	17	575	9, 991	5. 76
"	Selected [1] (Incubator)	9	201	2, 553	7. 87
"	Selected (Incubator)	12	176	3, 245	5. 42
"	Isogenic	16	167	6, 472	2. 58

[1] Observations previous to isolation of isogenic strain.



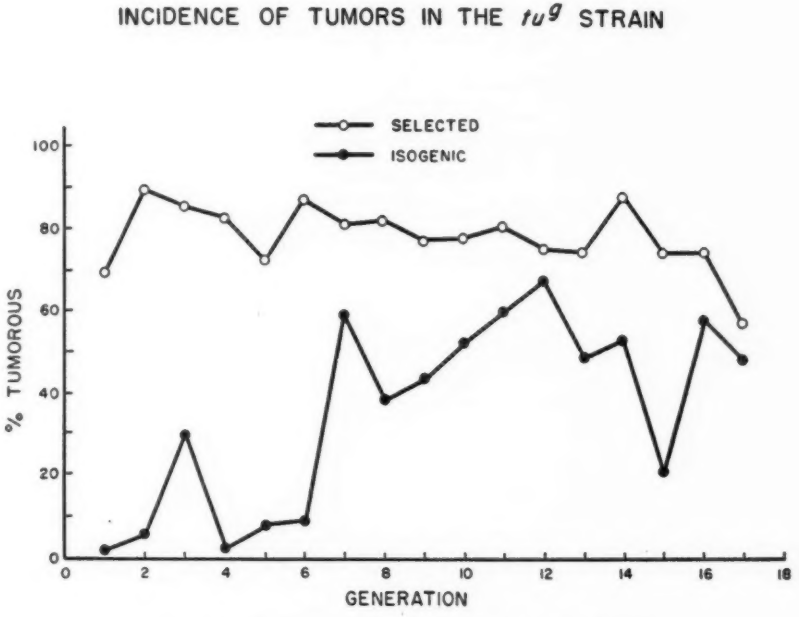
TEXT-FIGURE 2.—Incidence of tumors in the *tu 36 a* strain.

TABLE 2.—*Incidence of tumors in tu ' strain*

Sex	Group	Number of generations	Tumor-bearing	Total	Percent tumor-bearing
Male	Selected	9	3, 797	4, 370	86. 89
"	Isogenic	8	761	1, 479	51. 45
Female	Selected	9	2, 852	4, 394	64. 91
"	Isogenic	8	525	1, 253	41. 90
Both	Selected [1]	9	5, 046	8, 051	62. 68
"	Selected	33	15, 673	20, 671	75. 82
"	Isogenic	27	1, 973	6, 520	30. 26

[1] Observations previous to isolation of isogenic strain.

is lower in the isogenic than in the selected strains either before ($P<0.0001$) or after ($P<0.0001$) isolation of the isogenic strain (table 2 and text-fig. 3).



TEXT-FIGURE 3.—Incidence of tumors in the *tu*^{g} strain.

It is clear that the incidence of tumors in these isogenic strains is not only not increased but is actually lower than the incidence of tumors in the selected stock from which each originated. The gradual return of the percentage incidence in the isogenic strain toward that of the selected strain (text-figs. 2 and 3) cannot be explained satisfactorily, unless selection of germinal mutations for susceptibility is occurring. These results are similar to those for inbred strains with respect to the incomplete penetrance of genes for tumor susceptibility. Since all flies are not tumor-bearing in these stocks where all genes including genes for susceptibility to tumors are homozygous, then the expression of these tumor genes must be modified by extrinsic or intrinsic factors that are not constant.

SUMMARY

The *tu 36 a* and *tu*^{g} tumor strains of *Drosophila* showed a diminished incidence of tumors when isogenic strains were established and compared to selected stocks over numerous generations.

The difference in incidence of tumors between the two sexes was retained when an isogenic strain of the *tu*^{g} stock was established.

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CYTOLOGIC STUDY OF THE GASTRIC ASPIRATE FOR CARCINOMA CELLS: PARAFFIN-BLOCK TECHNIC [1] [2]

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From July 1947 to November 1948, a study of the value of examination of the gastric aspirate for carcinoma cells using the Papanicolaou technic was made in this hospital (*1–3*). The results of that study are summarized in table 1, together with certain statistics from the large series of other investigators as reported in the recent literature (*3–8*). The standard Papanicolaou technic has yielded a positive diagnosis in 23.8 percent to 73.9 percent of known gastric-carcinoma cases, and a false-positive diagnosis in 2.3 percent to 7 percent of cases without apparent gastric carcinoma. At the University of Michigan hospital the routine Papanicolaou technic has given a positive diagnosis in 26.5 percent of 74 patients with a clinical diagnosis of gastric neoplasm (including 42.9 percent positive diagnoses in 42 patients with histologically proved gastric carcinoma), and a false-positive diagnosis of 5.4 percent in patients without detectable gastric carcinoma.

Swarts *et al.* (*6*), in reporting on the Papanicolaou technic (table 1), state that some specimens were also prepared by fixing in formalin, embedding the sediment in paraffin, and staining sections with EA36 or hematoxylin and eosin. The results with the latter technic were found to parallel those with the routine Papanicolaou method. Richardson, Queen, and Bishop (*5*) prepared the gastric aspirate and lavage by fixing in formalin, precipitating with picric acid, embedding the sediment in paraffin, and staining sections with hematoxylin-orange G-eosin. This procedure gave 48.1 percent positive diagnoses in 27 cases of gastric neoplasm, and 3.9 percent false-positive diagnoses in 51 patients without gastric cancer. The chief advantage of this procedure lay in the preservation of cellular relationships, permitting better interpretation and a more reliable diagnosis.

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The present report is a study of gastric aspiration cytology using a paraffin-block technic and includes 631 aspirations in 525 patients, in the period from December 1948 to July 1950.

TABLE 1.—*Reported accuracy of gastric-aspiration cytology*

Author and method	With gastric neoplasm		Without gastric neoplasm	
	Cases	Percent positive	Cases	Percent positive
Fremont-Smith, Graham, and Meigs (*4*), 1948. (Papanicolaou technic)	65	54. 0	128	2. 3
Richardson, Queen, and Bishop (*5*), 1949. (Fixed in formalin, precipitated with picric acid, embedded in paraffin, sectioned, stained with hematoxylin-orange G-eosin)	27	48. 1	51	3. 9
Bryant, Craig, and Pollard (*3*), 1949. (Papanicolaou technic)[1]	[2] 74	26. 5	305	5. 4
	[3] 42	42. 9		
Swarts, Ragins, Bernstein, and Meyer (*6*), 1950. (Papanicolaou technic; also some prepared by fixing in formalin, embedding in paraffin, and staining with EA36 or H&E)[4]	[3] 67	23. 8	99	7. 0
Botsford and Tucker (*7*), 1950. (Papanicolaou technic)	23	73. 9	101	4. 0
Seybolt, Papanicolaou and Cooper (*8*), 1951. (Papanicolaou technic)	168	32. 8	707	2. 5
Present series (paraffin-block technic)	[2] 51	19. 0	474	1. 1
	[3] 43	20. 7		

[1] Figures adapted from reference (*5*) to show effect of inclusion of unsatisfactory specimens.
[2] Includes cases with clinical or histologic evidence of gastric neoplasm.
[3] Includes only cases with histologic proof of gastric neoplasm.
[4] Results of paraffin-block technic paralleled those with routine Papanicolaou technic.

METHOD

Following an overnight fast, the gastric contents were aspirated with a standard Levine tube and the stomach then lavaged with 50 cc. of physiological saline or Ringer's solution. These specimens were immediately fixed in an equal amount of 15 percent formaldehyde and centrifuged. In some cases it was necessary to add 95 percent alcohol in order to precipitate the mucus present. The sediment was embedded in paraffin in the usual manner for tissue study (*9*). Sections were then cut and stained with hematoxylin and eosin, and examined microscopically for carcinoma cells.

From the viewpoint of the pathologist, this technic has several definite advantages. The material is concentrated and examination is, therefore, easier and requires less time than with smears. There is clearer differentiation of cells, and problems involving excessive thicknesses of smeared material do not occur. Cellular relationships may be preserved; this is of particular value when small bits of tissue amounting to minute biopsies are aspirated.

RESULTS

Gastric carcinoma.—Sixty-three aspirations were done in 51 patients with a clinical or histologic diagnosis of gastric carcinoma. This group

included 53 aspirations in 43 patients with histologically proved gastric carcinoma, and 10 aspirations in 8 patients in whom the diagnosis of gastric carcinoma was established solely on a clinical basis. In the latter group of patients, the diagnosis was based on the general clinical course, together with characteristic X-ray evidence of gastric malignancy, and in most cases confirmatory gastroscopic findings.

In the total group of 51 cases of gastric carcinoma, aspirations were positive in 19.0 percent, suspicious in 14.9 percent, negative in 42.9 percent, and unsatisfactory in 23.8 percent (table 2). Included in this

TABLE 2.—*Aspiration results in patients with gastric carcinoma; paraffin-block technic*

	Percent positive	Percent suspicious	Percent negative	Percent unsatisfactory
Clinical or histologic diagnosis: 63 aspirations in 51 patients	19. 0	14. 9	42. 9	23. 8
Histologically proved diagnosis: 53 aspirations in 43 patients	20. 7	15. 1	39. 6	24. 5

group are the 43 patients with histologic proof of carcinoma of the stomach; in these, aspirations were positive in 20.7 percent, suspicious in 15.1 percent, negative in 39.6 percent, and unsatisfactory in 24.5 percent. When the results of the paraffin-block technic in this latter group of patients are compared with the results of the standard Papanicolaou technic in the 42 cases of histologically proved gastric carcinoma previously reported from this institution (*3*), the standard Papanicolaou technic is seen to yield positive diagnoses in over twice as many cases.

Apart from statistical analysis, and as a result of personal practical use, the block technic is favored by many experienced pathologists. Certainly, positive diagnoses by this method, especially when based on aggregates of cells in undisturbed relationship, carry a degree of certainty seldom attained by the use of smears. It is important, therefore, to discover why, in the present series, the method proved inferior to the Papanicolaou procedure. Of minor importance is the fact that the comparison is based on two different groups of patients. It is more probable that too much reliance was placed on the concentration of the material in the block. It was believed that the examination of a few sections would give a cellular volume comparable to that of the entire area of a smear. Apparently, this is not true. Probably enough step sections (interrupted series) through the block should be examined to provide an area equal to that of the usual smear. The results of the present study should not be considered as condemning the block method without further study of this factor.

Factors influencing positive and unsatisfactory examinations in known carcinoma cases.—Swarts *et al.* (*6*) reported unsatisfactory cytologic examination in 49 percent of 67 cases of gastric cancer, and associated these results with large tumor with extensive necrosis, obstruction at the

cardia or pylorus, or very anaplastic cases. Bishop (*10*) has stated that the technic [of Richardson, Queen, and Bishop (*5*)] tends to miss large, obstructing, necrotic-surface cancers in which the exfoliative cells are already in a process of necrosis, and scirrhous carcinomas without mucosal involvement. Graham, Ulfelder, and Green (*11*) state that in the advanced tumor with ulceration and necrosis only occasional recognizable cells may be desquamated, and they also comment on the inability of scirrhous carcinoma without mucosal involvement to shed cells into the lumen. Papanicolaou (*12*) attributes the high percentage of false-negative examinations in gastric cancer to rapid deterioration of cells in gastric juice and poor preservation in 15 percent of specimens, to lack of exfoliation in scirrhous carcinomas that do not usually reach the mucosal surface, and to the frequent presence of swallowed foreign cells and food particles.

In the present series we have attempted to correlate the cytologic report (*i. e.*, positive, suspicious, negative, or unsatisfactory) in gastric carcinoma with the factors of 1) retention of any degree as shown by roentgenologic examination of the stomach, 2) presence or absence of free hydrochloric acid on histamine-stimulated gastric analysis, and 3) ulceration of the neoplasm. These observations are summarized in table 3.

TABLE 3.—*Factors influencing diagnostic accuracy of gastric aspiration in carcinoma patients*

	Percent positive	Percent suspicious	Percent negative	Percent unsatisfactory
With retention (24 patients)	10. 7	17. 9	28. 6	42. 9
Without retention (27 patients)	25. 7	11. 4	54. 3	8. 5
With ulceration (29 patients)	16. 7	16. 7	44. 4	22. 2
Without ulceration (22 patients)	22. 2	11. 1	40. 7	25. 9
With free acid (17 patients)	9. 1	22. 7	50. 0	18. 1
Without free acid (26 patients)	21. 9	12. 5	40. 6	25. 0

It is realized that the small groups involved in this analysis do not permit statistically significant conclusions; this material is presented merely to indicate trends of correlation.

Of these factors, the presence of retention of any degree appeared to be the most important condition resulting in unsatisfactory examinations. In cases with retention, an unsatisfactory examination resulted in 42.9 percent and a positive diagnosis in 10.7 percent, but in cases with no retention, a positive diagnosis occurred in 25.7 percent, and an unsatisfactory examination was reported in only 8.5 percent. The presence of free hydrochloric acid was correlated with a positive diagnosis in only 9.1 percent, but achlorhydria was associated with a positive diagnosis in 21.9 percent. This is logical, since the presence of free hydrochloric acid would favor peptic digestion of cells present in the gastric fluid, particularly in the residuum specimen, and especially if there should be any delay in

fixing the aspirate in formalin. Ulceration of the surface of the neoplasm apparently did not affect the aspiration results. The combination of achlorhydria and absence of retention appeared to be a favorable situation for accurate diagnosis, yielding a positive diagnosis in 29.4 percent and an unsatisfactory examination in only 5.9 percent.

Scirrhous carcinoma of the stomach was present in 7 patients in this series, giving a positive aspiration in 12.5 percent. The low diagnostic accuracy is attributed in part to the infrequent mucosal involvement with this type of carcinoma.

False-positive reports.—A total of 568 aspirations was done in 474 patients without clinical evidence of gastric neoplasm (table 4). Of these

TABLE 4.—*Aspiration results in patients without gastric carcinoma; paraffin-block technic*

[568 aspirations in 474 patients]

	Cases positive	Cases suspicious	Cases negative	Cases unsatisfactory
Without gastrointestinal symptoms (314 cases)	2	11	280	36
With gastrointestinal symptoms (160 cases)	4	29	181	25
Total	6	40	461	61
Percent	1.1	7.0	81.2	10.7

patients 160 had gastrointestinal symptoms of some type, and 314 had no such symptoms. A false-positive cytologic diagnosis of carcinoma was reported in 6 patients, or 1.1 percent of the total group. In 305 patients without clinical gastric carcinoma previously studied by the Papanicolaou method, the incidence of false-positive diagnoses was 5.4 percent. In the present series the 6 false-positive reports occurred in 3 patients without detectable disease of the gastrointestinal tract, in 1 patient with hypertrophic gastritis, and in 2 patients with apparently benign gastric ulcer. In 76 patients with a clinical diagnosis of benign gastric ulcer, a false-positive cytologic diagnosis was reported in only these 2 cases.

In respect to both series, it must be recognized that sufficient time has not elapsed to establish with certainty that silent subclinical carcinoma may not be present. Positive diagnoses in this category must be considered as apparently false in the light of all available information.

Gastric aspiration as a survey procedure for gastric carcinoma.—Included in the present series are 331 aspirations in 315 adult hospitalized patients who had no gastrointestinal symptoms of any type, but who were subjected to this examination in an attempt to evaluate gastric aspiration as a survey procedure for gastric carcinoma. One case of unsuspected gastric carcinoma was discovered as a result of this survey.

L. M., a 79-year-old white male, was admitted to the urology service of University Hospital on February 2, 1949, because of intermittent urinary dribbling of 8 years' duration. Repeated questioning disclosed no gastrointestinal symptoms. Physical examination was negative except for benign prostatic hypertrophy. The blood hemoglobin was 11.5 gm. On February 4, 1949, the patient underwent retropubic

prostatectomy and recovered uneventfully. During this hospitalization a gastric aspiration was examined for carcinoma cells as a part of a survey of patients without gastrointestinal symptoms. This showed "carcinoma cells probably of gland cell type." The patient was advised to have X-ray examination of the stomach and gastroscopy but refused these procedures, pointing out that he had no digestive-tract symptoms. He left the hospital on February 17, 1949, but returned for readmission on June 27, 1949, with symptoms of progressive dysphagia, ease of fatigue, and vague epigastric distress. Physical examination showed evidence of recent weight loss. The blood hemoglobin was 11.0 gm. Gastric analysis showed no free acid on histamine stimulation. X-ray examination of the stomach showed an intrinsic neoplasm. Gastroscopy was unsuccessful. On June 30, 1949, a total gastrectomy and esophagojejunostomy were done for a poorly differentiated gland-cell carcinoma arising in the gastric mucosa. Metastases were present in regional lymph nodes and the mesocolon. The patient died 2 months later from metastatic carcinoma.

The refusal of this patient to undergo further diagnostic studies or treatment until his cancer became symptomatic is similar to the experience of Roach, Sloan, and Morgan (*13*); in studying photofluorography of the stomach as a survey procedure for gastric cancer, they discovered 3 patients with unsuspected gastric neoplasms in 2,500 examinations, but all 3 patients refused operation because of lack of symptoms.

The ability of cytologic study of the gastric aspirate to detect early as well as advanced neoplasm has been confirmed by the experience of several authors. Richardson *et al.* (*5*) reported a positive diagnosis in one patient having a gastric cancer 1 mm. in diameter. Papanicolaou and Cooper (*14*) reported a positive aspiration in a patient with a gastric ulcer, which on microscopic study showed a small cancer in one quadrant of the ulcer. Fremont-Smith *et al.* (*15*) reported a study that included five "early" cases of gastric carcinoma, four having positive aspirations. Nevertheless, because of the low incidence of positive aspirations in known carcinoma cases, gastric-aspiration cytologic study is not an acceptable survey procedure with any of the technics commonly used. Several variations of the aspiration technic itself have been suggested for the purpose of obtaining cells better preserved for examination (*16–19*), but most of these procedures involve unusual maneuvers or apparatus and have not yet been widely adopted.

SUMMARY

In 51 patients with a clinical diagnosis of gastric carcinoma, including 43 patients with histologic proof, positive aspirations were obtained in 19.0 percent and 20.7 percent, respectively, using a paraffin-block technic. This incidence of positive results is approximately half that obtained with the standard Papanicolaou technic in the histologically proved cases previously reported from this hospital. It is believed that the difference is due to the smaller amount of cellular material examined in a limited number of sections through a small block. False-positive examinations were reported in 1.1 percent of 474 patients without apparent gastric neoplasm; with the Papanicolaou method, false-positive examinations were reported in 5.4 percent.

In patients with known gastric carcinoma, one of the important factors

associated with unsatisfactory examinations appeared to be gastric retention of any degree. A favorable situation for accurate diagnosis was the combination of achlorhydria and absence of retention.

This study included a survey of 315 adult hospitalized patients with no gastrointestinal symptoms of any type. One unsuspected gastric carcinoma was discovered in this group. Although cytologic examination of the gastric aspirate is capable of detecting early carcinoma in some patients, we do not consider this technic, used alone, to be a satisfactory survey procedure because of its low diagnostic accuracy in known carcinoma patients.

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FAILURE OF EUGENOL AND HEAT TO POTENTIATE GASTRIC TUMOR INDUCTION BY 20-METHYLCHOLANTHRENE IN MICE [1] [2]

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Orally administered 20-methylcholanthrene, or other carcinogenic hydrocarbons (*2, 20, 33, 34*), are incapable of inducing adenocarcinomas in the glandular mucosa of the stomach of the mouse. Recently, we have attempted to potentiate the action of the carcinogen with physical or chemical additives.[5] Hollander (*13*) has shown eugenol emulsion to be a satisfactory chemical desquamator for the gastric mucosa, by exposing gastric pouches of dogs to eugenol for 15 minutes and noting breakdown of the mucous barrier with numerous desquamated cells in the secretions. Histologic examination showed depletion of the mucous cells and destruction of crypts in the epithelium. Two hours after eugenol had been applied, the mucosal surface was markedly denuded. Thirty-six hours later there was regeneration, and crypts were reappearing (*13*).

An olive-oil emulsion of eugenol was used here to penetrate the mucous barrier of the mouse stomach, and create a condition of regeneration with actively proliferating cells; this active mucosa might be a good medium upon which the methylcholanthrene could act. Since it was necessary to intubate the mice in administering the eugenol emulsion, it was deemed pertinent also to administer the carcinogen by tube heated to the highest temperature tolerated internally by mice, and obtain a combined action of chemical trauma, physical trauma, and carcinogenic action.

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[5] Methylcholanthrene was supplied by the Eastman Kodak Research Laboratories, and was blended into an emulsion as reported by Lorenz. To 100 cc. of light mineral oil was added cetyl alcohol, 5 gm.; glyceryl monostearate, 6 gm.; cholesterol, 1 gm.; methylcholanthrene, 400 mg.; aerosol O. T., 70 percent solution, 5 gm. The ingredients were mixed at 70°-90° C., and poured into 300 cc. of distilled water; after brief shaking the volume was brought to one liter and the pH adjusted to 9.

MATERIALS AND METHODS

Mice of strains C3H, A, and I, were housed in double-compartment wooden cages with five animals to one compartment. Purina fox-chow pellets were fed *ad libitum*, and drinking water was constantly available. Mice were intubated periodically with a blunted 18-gauge needle provided with a slight curve in the shaft. It was possible to pass this tube into the stomach of a mouse with minimal mechanical trauma, once the technic was mastered. A maximum of 52 injections of animals in the experimental groups was made; 18 eugenol and olive-oil instillations (25 percent) plus 34 carcinogen-emulsion instillations. The injections covered a period of 191 days, with an average of 3.6 days between injections. Usually, however, a eugenol injection was followed the next day by a carcinogen injection.

One group of mice received 0.2 cc. of carcinogen emulsion heated to 65° C., and the other received the same emulsion at room temperature. Previous studies by the author, using a thermocouple in the stomach of the mouse and a string-gauge galvanometer to record deflections, showed a temperature drop of 2°–4° C. from the constant water bath to the stomach.

Mice were examined weekly for palpable abdominal tumors, and undue weight loss was a criterion for sacrificing them. Animals were killed by cervical dislocation, and the stomach was distended with 10 percent formalin fixative. Specimens of liver, lung, kidney, and lymph nodes were also preserved. Microscopic sections, mounted in paraffin, were stained with hematoxylin-eosin.

Mice were deferred from 8 a. m. until late afternoon on the days of intubation to empty the stomachs sufficiently. Animals receiving hot carcinogen emulsion reacted considerably to instillation of the material in the stomach; rate of respiration was markedly increased for 4 or 5 minutes, and the mice would frequently convulse a number of times. Animals receiving cool carcinogen emulsion rarely manifested more than a rapid respiratory rate for a minute or two.

RESULTS

Several acute experiments were performed to determine the effect of eugenol on the mucosa of the stomach. Of full strength eugenol, 0.2 cc. produced marked desquamation of cells in the prepyloric mucosa. Figure 1 represents a histologic section of glandular mucosa made ½ hour after 0.2 cc. eugenol had been instilled into the stomach of the mouse. The surface epithelium is severely traumatized, and crypts are destroyed to ½ the depth of the mucosa.

Six squamous-cell carcinomas of the forestomach resulted; five in the mice receiving cool carcinogen (4 strain A, 1 strain C3H) and one in the group receiving hot carcinogen (strain A) (table 1). No adenocarcinomas were found. Several cancerous stomachs had benign papillomas in addition to the malignant growth, and one stomach had a single benign squamous papilloma.

TABLE 1.

Group	Total number mice	Number squamous cancers	Average injections eugenol	Average injections carcinogen	Average number days on experiment	Average number days on experimental animals with lesions
Cold carcinogen	27	5	13	25	205. 3	371
Hot carcinogen	31	1	12	24. 9	169. 7	434
Eugenol controls	15	0	15	0	250. 0	0

Four squamous lesions in strain A mice were diagnosed "nearocarcinoma" (after Stewart) but were not included in the figures for malignant lesions. Three of the "nearocarcinomas" appeared in the mice intubated with hot carcinogen, and one in the group given cool carcinogen. If these lesions were added to the frankly malignant tumors there would be six cancers with cool carcinogen and four cancers with hot carcinogen. The marked disparity between the groups is thereby considerably lessened. Nine liver specimens demonstrated toxic effects from eugenol with pale, vacuolated hepatic cord cells and granular cytoplasm. Sixteen deaths were directly attributable to aspiration of intubated material, and this represented 21 percent of the total number of mice.

The squamous-cell carcinomas are well advanced lesions, infiltrating to the serosa (fig. 6) and frequently extending to adjacent organs, as the liver in figure 4. A markedly cellular, hyperchromatic lesion is noted in figure 2, where anaplasia and disorganization are prominent features in the diagnosis of malignant disease.

DISCUSSION

The physical factor, heat, and the chemical factor, eugenol, seemingly did not potentiate the effect of methylcholanthrene upon the glandular mucosa. In 1945, Ivy (*14*) presented evidence that perfusion of a dog's gastric pouch with 100 cc. of water and hydrogen peroxide heated to 50°–60° C. would produce an effect of acute gastritis. Klein and Palmer (*16*) in 1940, raised the question of heat having importance in esophageal and gastric cancer in man, especially since Lerche pointed out in 1916 that carcinomas of the esophagus and stomach tend to predominate in the regions just proximal to the points of physiologic narrowing. The possibility of a prolongation of the effect of the heat at these sites was postulated.

The author intubated 44 mice of strains C3H and A several years ago for a 14-month period, using water at a temperature of 65° C. as it entered the stomach of the mouse. A total of 112 esophageal injections was made, and 31 mice received 75 or more instillations of water heated to 65° C. at 3-day intervals. Histologic study of the esophagus and stomach of these mice failed to demonstrate any effects from the multiple instillations.[6]

[6] Unpublished data.

Perhaps future experiments wherein mice would be fed a balanced diet containing methylcholanthrene, and intubated with eugenol, Papain, or lysozyme every 2 or 3 days, would be more successful by permitting longer action of the carcinogen upon the exposed or regenerating mucosa.

SUMMARY

Mice of strains C3H, A, and I received eugenol in olive oil and methylcholanthrene emulsion on alternate days by stomach tube.

One group of mice was given the carcinogen emulsion heated to 65° C. The second experimental group received cool, room-temperature emulsion. The controls received eugenol emulsion only.

Five squamous-cell carcinomas of the proventriculus resulted in the cool carcinogen group, and one squamous-cell lesion appeared in the hot carcinogen group. No adenocarcinomas were produced.

The combined effects of heat (65° C.) and eugenol failed to potentiate the effect of methylcholanthrene upon the glandular gastric mucosa of mice.

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PLATE 69

FIGURE 1.—Glandular mucosa of mouse stomach sectioned ½ hour after 0.2 cc. of 25-percent eugenol was instilled by intubation. Note depth of cellular trauma with the destruction of crypts and extensive desquamation of cells. × 110

FIGURE 2.—Photomicrograph of squamous-cell cancer of proventriculus in a C3H mouse that received 18 eugenol injections and 34 cool carcinogen emulsion injections. Mouse died 434 days after start of intubation. × 40

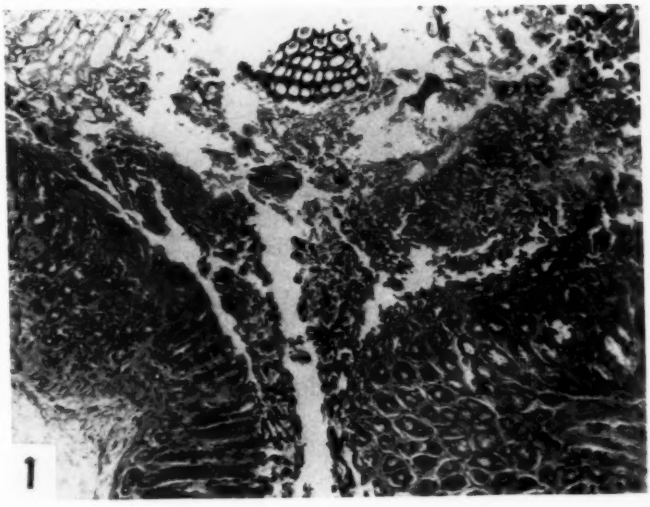

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PLATE 70

FIGURE 3.—Stomach of strain A female mouse (EA_{40}) with 18 eugenol injections, and 34 cool carcinogen injections. Death 208 days after start of injections. Serosal surface right; mucosa, left. Circa × 4

FIGURE 4.—Photomicrograph of lesion in figure 3, showing direct extension of tumor cells to the liver. × 30

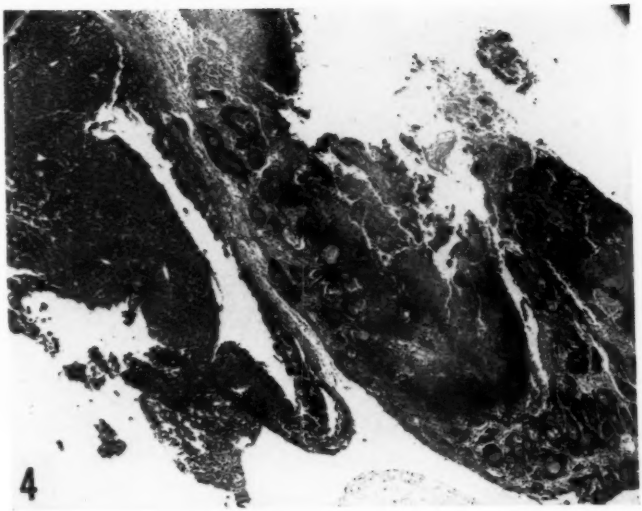

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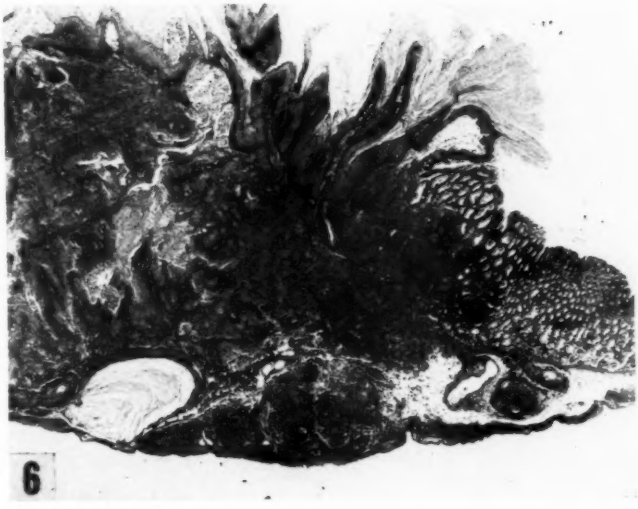
PLATE 71

FIGURE 5.—Stomach of strain A female mouse (EA_{11}) with 18 eugenol and 34 hot carcinogen injections. Death 434 days after start of intubations. Lesion encroaching upon limiting ridge and larger lesion at apex of proventriculus, along greater curvature, are malignant. Circa × 4

FIGURE 6.—Photomicrograph of lesion at limiting ridge in figure 5. Note cellularity and invasion into glandular mucosa. × 30



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THE ACTION OF METHYLCHOLANTHRENE, CROTON OIL, AND 1,2-BENZANTHRACENE IN THE INDUCTION OF SKIN TUMORS IN STRAIN DBA MICE[1,2]

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In 1941 (*1*), it was reported that croton oil, a noncarcinogen for the skin of mice, enhanced the tumorigenic potency of low doses of a carcinogen when both were applied percutaneously. In a later investigation, significantly more skin tumors were obtained among mice painted with 3,4-benzpyrene if these animals were subsequently painted with croton oil. At the same time, croton oil was reported to enhance the transformation of benign warts to malignant tumors (*2*). These observations implied that different processes were involved in the initiation and development of skin tumors in mice. The experiments of Rous and his associates (*3–5*) with skin tumors in rabbits led to similar conclusions. It was observed that tar papillomas that regressed, subsequently reappeared following the application of a noncarcinogenic agent, such as turpentine, or wound-healing. To account for these results, two stages in skin tumorigenesis were proposed: initiation, when "latent tumor cells" are produced, and promotion, when these cells are stimulated to develop into visible tumors (*5*).

When Mottram (*6*) demonstrated that one painting with a carcinogen followed by croton oil elicited skin tumors in mice, a method was at hand for a quantitative analysis of initiation and promotion. With this technic, Berenblum and Shubik (*7*) observed that when mice were painted with different carcinogens or with different concentrations of the same carcinogen and received equal amounts of croton oil, tumor incidence varied but latent period remained unchanged. When a fixed concentration of one carcinogen was employed and the time between its administration and the start of croton-oil painting was delayed, latent period was correspondingly delayed but tumor incidence did not vary significantly.

According to Shubik (*8*), most skin tumors induced in the mouse by one painting with a carcinogen followed by croton oil are benign. Similar results have been reported by other investigators (*9*, *10*).

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[2] The technical assistance of Frank M. McCathran is gratefully acknowledged.
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[4] National Institutes of Health, Public Health Service, Federal Security Agency.

Experiments in this laboratory with strain DBA mice in which the cocarcinogenic activity of croton oil following a single application of methylcholanthrene was investigated, yielded results in essential agreement with those of others (*7, 11*), although the tendency for papillomas to develop into carcinomas was not observed.

The hydrocarbon 1,2-benzanthracene, which is closely related to methylcholanthrene, has been observed repeatedly to possess little or no tumorigenic activity (*2, 12–15*). In a recent investigation, however, it was reported that 1,2-benzanthracene in tricaprylin induced sarcomas in C57 black mice following subcutaneous injection. Injection of the solvent alone also produced sarcomas (*16*). If 1,2-benzanthracene is noncarcinogenic, or weakly so, this could be due to a lack of initiating or promoting power, or both. Perhaps 1,2-benzanthracene would be active in skin tumorigenesis once "latent tumor cells" had been induced or after development of visible benign tumors. Investigation of both these possibilities disclosed that 1,2-benzanthracene was unable to affect tumorigenesis in the skin of the mouse at the stages examined. Details of these experiments are reported in this paper.

MATERIALS AND METHODS

Strain DBA male mice, subline 212, 7 to 17 weeks of age and raised at the National Cancer Institute, were used in these experiments. They were housed either in wooden boxes 28 by 18 by 15 cm., or in plastic ones 28 by 18 by 12 cm., no more than 8 animals to a cage. All mice were maintained at a temperature of 76°–78° F., and were supplied with an unlimited amount of Purina laboratory chow and tap water.

20-Methylcholanthrene (m. p. 179°–180° C.)[5] hereafter referred to as MCA, was prepared as a 0.5-percent solution, 1,2-benzanthracene (m. p. 159°–160° C.)[6] hereafter referred to as BA, as a 0.4 percent solution, and croton oil[7] as a 5-percent solution. The solvent in each instance was olive oil, U. S. P. grade. Where BA and croton oil were applied together, a 0.4 percent solution of the hydrocarbon was prepared in a 5-percent solution of croton oil in olive oil.

All solutions were applied percutaneously with a No. 3 camel-hair brush. Two different sites were used: the skin at the nape of the neck and the inner surface of both ears. Prior to painting the neck region, the fur was clipped with fine scissors. Clipping was repeated as necessary throughout the experiment. Mice in this group received approximately 0.2 to 0.3 ml. of solution per application. Where the ears of mice were painted, each ear received approximately 0.02 to 0.03 ml. of solution.

Mice were painted once with the MCA solution and kept one to a cage for 4 to 5 days after which they were housed eight to a cage. This was to minimize loss of carcinogen through licking and rubbing. Two weeks after the application of MCA, routine paintings were commenced with the

[5] Obtained from Edcan Laboratories, South Norwalk, Conn.
[6] Obtained from Eastman Kodak Co., Rochester, N. Y.
[7] Obtained from Fisher Scientific Co., Eimer and Amend, New York, N. Y.

other solutions. These were continued three times weekly throughout the experiment until the animals died or were sacrificed. Among the mice painted with croton oil, toxic symptoms sometimes appeared. At such times, the affected mice received only one or two applications per week for 3 to 4 weeks, when they again were painted three times weekly.

The mice were examined once weekly for tumors. Papillomas were recorded from the time they reached a diameter of 1 mm., but only when they persisted 2 weeks or longer. At autopsy, all excised tissues were fixed in Tellyesniczky's fluid, sectioned at 8 micra, and stained with hematoxylin and eosin. Tumor diagnoses were confirmed following microscopic examination of these tissues.[8]

EXPERIMENTAL PROCEDURES AND RESULTS

A total of 352 mice divided into 8 groups was used in these experiments. In the first 3, Series A–C, solutions were applied to the nape of the neck; while in the remainder, Series D–H, the ears were painted. A list of the different experimental groups and the treatment for each follow:

Series A–40 mice, painted with 5 percent croton oil in olive oil.

The mice in Series B–H inclusive were painted once with the MCA solution. Two weeks thereafter, painting with other solutions was begun as follows:

Series B–40 mice. No additional treatment.
Series C–40 mice, painted with 5 percent croton oil in olive oil.
Series D–40 mice. No additional treatment.
Series E–40 mice, painted with olive oil alone.
Series F–56 mice, painted with 5 percent croton oil in olive oil.
Series G–48 mice, painted with a 0.4 percent solution of BA in olive oil.
Series H–48 mice, painted with 0.4 percent BA and 5 percent croton oil in olive oil.

The results of these experiments have been summarized in table 1. No skin tumors were observed in Series A during a mean period of observation 252 days. Despite an absence of neoplasms, histologic examination of the painted skins revealed a marked, generalized, epithelial hyperplasia.

In Series B, no macroscopic skin tumors were observed, nor were any discovered following histologic examination. The mean period of observation of the mice in this Series was 264 days, at which time the epidermis appeared normal histologically.

In Series C, 27 of 34 mice (79 percent) developed tumors during a mean period of observation of 177 days. Fifty percent of all the tumor bearers had tumors by the fifty-ninth day. These mice received an average of 53 croton-oil applications compared with 90 for those in Series

[8] The author wishes to thank Dr. Thelma B. Dunn of the Laboratory of Pathology of the National Cancer Institute or her assistance.

TABLE 1.—*Percutaneous administration of solutions of 20-methylcholanthrene, 1,2-benzanthracene, croton oil, and olive oil in strain DBA male mice (except for series A, the mice were painted once with a 0.5 percent MCA solution. Two weeks thereafter, application of other solutions was begun)*

Series	Survivors at time of first tumor	Treatment	Site	Total applications	Mice with papillomas	Latent period [1]	Total papillomas	Average age of mice		Mean period of observation
								At start	At death	
	Number	*Solution*		*Average number*	*Number*	*Days*	*Number*	*Days*	*Days*	*Days*
A	34	5 percent croton oil in olive oil	Nape of neck	90	0			105	357	252
B	35	None	do	0	0			51	315	264
C	34	5 percent croton oil in olive oil	do	53	27	59	60	53	230	177
D	38	None	Ears	0	0			69	398	329
E	40	100 percent olive oil	do	120	0			71	378	307
F	51	5 percent croton oil in olive oil	do	70	35	89	63	77	299	222
G	41	0.4 percent BA in olive oil	do	70	0			116	318	202
H	42	0.4 percent BA and 5 percent croton oil in olive oil.	do	60	28	65	73	111	315	204

[1] Latent period—time when 50 percent of all the tumor bearers in the series concerned had tumors 1 mm. or more in diameter.

A. A total of 60 tumors was observed in Series C, an average of 2.2 per tumor bearer. All these neoplasms were benign papillomas.

Among the 38 survivors in Series D that had been painted once with MCA, no macroscopic tumors appeared after a mean period of observation of 329 days. None was revealed by histologic examination of ear sections.

The 40 DBA males in Series E received an average of 120 olive-oil paintings during a mean period of observation of 307 days. A 1-mm. skin papilloma appeared in 1 of the mice. However, since this mass regressed within 2 weeks, it was not included in the data. No epidermal hyperplasia was observed microscopically in the ear tissues of this Series.

A 69 percent tumor incidence was obtained in Series F during a mean period of observation of 222 days. In all, 63 benign papillomas were observed, an average of 1.8 per tumor bearer. Fifty percent of all the tumor bearers had tumors within 89 days from the start of painting. Histologically, the nontumor-bearing sections of ear from Series F disclosed widespread epidermal hyperplasia.

The 41 survivors in Series G received an average of 70 paintings but no skin tumors were discovered during a mean period of observation of 202 days. Upon histologic examination of ear sections in this Series, intact epidermis with little or no evidence of hyperplasia was observed.

Twenty-eight of 42 mice (67 percent) in Series H had skin tumors during a mean period of observation of 204 days. A total of 73 neoplasms was observed among the 28 tumor bearers. Fifty percent of all the tumor bearers had tumors by the sixty-fifth day of painting. Extensive epidermal hyperplasia was observed among the ear sections examined both from the tumor-bearing and nontumor-bearing mice.

No malignant tumors were observed in any of the mice. Benign papillomas continued to arise throughout the experiment, and their growth rates varied appreciably. Some enlarged slowly to about 2 mm., survived for several weeks, then dried up and sloughed. Others enlarged similarly but remained. Some benign papillomas with greater growth potential continued to enlarge to a height of 6 or more mm. Frequently, these had a cauliflower-like aspect and appeared more fleshy than the slower growing sessile tumors. Regressions, which were common among the slow growing papillomas, were observed less commonly among the more rapidly growing ones. In experiments of this type, Shubik (*8*) has described, in more detail, observations similar to these. In addition to the above tumors, two characteristic "frill horns," 1 cm. and 1.3 cm. in height, respectively, were observed in Series H.

DISCUSSION

From the data in table 1, it will be observed that continued application of croton oil to the skin of mice following one painting with MCA produced many papillomas. Croton oil alone yielded none and neither did MCA alone. Thus, croton oil exerted a definite promoting influence in

the genesis of skin tumors. This also had been observed when stock mice and mice of the Swiss and CF1 strains were employed (*6–8, 11*).

In the present experiments, solutions were applied either to the nape of the neck, clipped free of hair, or to the inner surface of the ears. Tumors appeared readily and about as frequently in both sites. Berg (*17*) reported that ear epidermis in strain D–Z mice was less sensitive than skin of the back to benzpyrene. Following painting of the back with this carcinogen, he observed a perinuclear fluorescence in the epidermal cells. This reaction was not regularly seen in epidermis of the ear. The 69 percent and 67 percent tumor incidences obtained in the mice treated on the ears (Series F, H), compared favorably with the 79 percent incidence in the mice treated on the back (Series C). Thus, in strain DBA mice, we did not observe a significant difference in tumor susceptibility for these two sites. In some respects, the mouse's ear is a better site than the back for the application of carcinogenic and/or other solutions. The routine task of hair removal is eliminated, and the painted area is clearly delimited. Tumors are discovered early and their growth charted easily. Finally, tumors may be treated or excised with a minimum of effort.

In the present investigation, none of the induced tumors had an observable tendency towards malignancy. The slower growing papillomas frequently had a tendency to regress, whereas this was less frequently observed among the rapidly growing ones. It had been reported (*8–11*) that croton oil possessed, at best, a limited ability to promote the development of skin carcinomas in mice painted once with a low dose of carcinogen. Thus, in one experiment (*8*), only 8 malignant lesions were observed out of a total of 400 tumors. Bielschowsky and Bullough (*10*) found that a large dose of MCA induced more carcinomas and at an earlier average age than a small dose. It has also been observed (*2, 18*) that when mice are painted a number of times with 3,4-benzpyrene or MCA and then continuously with croton oil that both benign papillomas and carcinomas are obtained. The latter experiments indicate that the amount of carcinogen administered is an important factor in the induction of malignant skin tumors, and may help to explain the absence of skin carcinomas following one painting with a small dose of MCA.

Berenblum (*2*) did not observe any skin tumors in mice that had received repeated percutaneous applications of BA alone or in solution with croton oil. In Series G, the action of BA on epidermis that had been painted once with MCA was investigated; no tumors were elicited, whereas in Series F, croton oil was employed and 63 papillomas were observed. While the results of Berenblum show that BA lacks the ability to induce skin tumors or to initiate the "latent tumor cell" change, the present experiments indicate, in addition, that BA is unable to promote the development of visible tumors after "latent tumor cells" have been produced. Many papillomas were observed among the mice of Series H, where a single application of MCA was followed by repeated paintings with BA and croton oil. Although the papillomas that arose in the treated area were painted along with the surrounding epidermis, this had no observable

influence in transforming any of them to malignant tumors. Also, no appreciable differences in regression rate, tumor incidence, tumor types, or latent period were obtained in Series H compared with Series F, where croton oil alone was employed.

The benign tumors that appeared throughout the experiment included rapidly growing as well as slowly growing papillomas. It had been suggested (*8*) that one painting with a carcinogen initially induced a host of "latent tumor cells," which vary in growth potential. Accordingly, one might have expected those tumors with greater growth potential to appear first, while others with less became visible later. This was not the case, for papillomas with varying growth rates appeared early as well as late. It may be as Friedewald and Rous (*19*) suggest—that epidermis treated with carcinogen results first in the production of cells with "latent neoplastic potentialities" and that these become neoplastic later "at widely various, evenly graded rates." If the tumors which then appear also vary in growth potential, it might help explain the results of the present experiments.

SUMMARY

Strain DBA mice were painted continuously on the ear or nape of the neck with 5 percent croton oil, or once with 0.5 percent 20-methylcholanthrene and then continuously with one of the following: 5 percent croton oil in olive oil, olive oil, 0.4 percent 1,2-benzanthracene in olive oil, 0.4 percent 1,2-benzanthracene and 5 percent croton oil in olive-oil solution, or nothing. Benign papillomas were induced readily in either site in mice that received both methylcholanthrene and croton oil, but not in others. Many of the tumors, especially those that grew slowly, regressed. Repeated painting of papillomas with croton oil alone or in solution with 1,2-benzanthracene resulted in no observable malignant tumors. Also, painting with 1,2-benzanthracene plus croton oil had no appreciable effect on tumor incidence, latent period, or growth rate as compared with croton oil alone.

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SPONTANEOUS PULMONARY TUMOR IN THE RAT: REPORT OF A LESION [1]

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The rarity of pulmonary neoplasms arising spontaneously in the rat has been observed and commented upon frequently in the literature of experimental medicine. Thus, no pulmonary tumors were found in a total of 142,768 autopsies on rats reported by McCoy (*1*), Ratcliffe (*2*), Woolley and Wherry (*3*), and Bullock and Rohdenburg (*4*). Bullock and Curtis (*5*, *6*) in a later study of spontaneous growths occurring in a large colony of rats made the observation that there existed a relative insusceptibility of these rats to neoplasia of the lung. While many of the rats dying in the "cancer age" exhibited lesions of chronic bronchopneumonia, primary tumors of the lung were rarely seen. Only 4 pulmonary tumors among 521 primary spontaneous tumors were recorded; these were an osteochondrosarcoma, a squamous-cell carcinoma, a polymorphous-cell tumor, and a fibrosarcoma. In an experiment designed to evaluate the relation of nutrition and rate of growth to life span, Saxton *et al.* (*7*) recorded 234 neoplasms in 184 of the 498 Osborne-Mendel rats studied. Each of 1 control and 1 underfed rat developed an adenoma of the lung, while 91 of these animals showed a lesion of reticulum-cell sarcoma in the lung. Thus it is apparent that, unlike the mouse, spontaneous pulmonary neoplasms occur infrequently in the rat.

The induction of lung tumors in rats by means of carcinogens has been moderately successful. Urethan administered by any of a number of routes produces pulmonary tumors in albino rats in an incidence of 60 percent of the animals treated (*8–10*). Orr and Bielschowsky (*11*) report the induction of lung tumors in 10 percent of rats of the Wistar and Piebald strains when treated with 2-acetylaminofluorene. These authors make the point that bronchiectasis, when extensive in old rats, might obscure the presence of tumor growths and thereby create a false impression as to paucity of spontaneous neoplastic lesions. Lisco and Finkel (*12*) reported in an abstract the administration of radioactive cerium as an aerosol to rats in an attempt to induce tumors. They suggested that malignant tumors arose from areas of bronchial metaplasia in these animals.

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

CASE REPORT

The tumor herein described occurred in a 12-month-old, untreated, stock male rat of the Marshall strain 520. The tumor appeared in the right lower lobe of the lung as a pale grey, wedge-shaped nodule 4 mm. in its greatest dimension, with the base toward the pleural surface. The nodule was slightly elevated above the lung surface. While not encapsulated, the tumor was sharply circumscribed and blended into the adjacent parenchyma (fig. 1). The lungs were fixed in Zenker's acetic fluid.

Microscopically, the tumor resembled the common pulmonary tumor of the mouse, displaying a papillary glandular pattern (fig. 2). The stroma was scant and composed of mature connective tissue, free of inflammatory cells and containing relatively few blood vessels. Study of the tumor with van Gieson and PTAH stains revealed fine bundles of collagenic fibers in the stroma. Silver impregnation (Wilder) demonstrated reticulum fibers confined to the stroma of the tumor and having a pattern resembling that of a papillary glandular growth. Most of the tumor cells were cuboidal while some appeared to be columnar, a few polygonal, and a very few flat. The cytoplasm was moderately abundant and, generally, deeply acidophilic. Periodic acid-Schiff preparation showed the stroma well outlined but no PAS-positive material was found in the tumor cells. The nuclei were fairly uniform in size; occasional nuclei were rounded and others, particularly those in the columnar cells, were elongated. The nucleus in the columnar cell tends to be basally situated but may occur in any part of the cell. Some of the nuclei were deeply hyperchromatic and had a heavily stained nuclear wall with several fine dustlike particles within the nucleus. There was usually a single large mass of chromatin; sometimes two or three such masses. Frequently a single acidophilic nuclear inclusion occurred. The cells of the tumor were well preserved and showed little or no evidence of degeneration except for those that were desquamated. An occasional tumor cell showed two or three nuclei, and often one of these was relatively large. In the stroma there were occasionally a few lymphocytes and a few large vacuolated macrophage-like cells. Study of the cells with the phosphotungstic-acid-hematoxylin stain showed no cilia. The lumens of many of the gland spaces were empty; others contained a granular material and a few desquamated cells. There was a bronchus at the apical margin of the deepest aspect of the tumor which gave rise to a bronchiole, the lining cells of which were continuous with the tumor cells. In the alveoli surrounding the tumor, the walls were often partially lined by the tumor cells in continuity with the main tumor nodule (figs. 3 and 4).

A section of the lung away from the tumor showed a minimal chronic inflammatory reaction around many of the bronchi and blood vessels. The latter showed pronounced edema of their connective-tissue coverings. There was no evidence of metastasis of this pulmonary tumor.

COMMENT

This lesion represents the only spontaneous pulmonary tumor in the rat seen in the material from this Institute. The frequency of pulmonary tumors in the rat precludes the study of the spontaneous lesion as has been done in the mouse but lends significance to the induction of this lesion by experimental methods even though these methods are limited. Morphologically, the pulmonary tumors of the rat and those occurring in the mouse are fundamentally similar. The spontaneous pulmonary tumor described herewith resembles other tumors in the rat reported in the literature except for those of the squamous type induced by Orr and Bielschowsky (*11*). The origin of this neoplasm cannot be deduced from a single tumor. It was observed, however, that no inflammatory reaction was seen in the pulmonary parenchyma regional to the tumor, nor was there any associated bronchiectasis.

SUMMARY

A pulmonary tumor occurring spontaneously in an untreated rat is reported.

The rarity of spontaneous lung tumors in rats as evidenced in the literature of experimental medicine is stressed.

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PLATE 72

FIGURE 1.—Entire tumor. Hematoxylin and eosin. × 30

FIGURE 2.—Higher-power view of tumor, showing papillary glandular structure of tumor. Hematoxylin and eosin. × 260

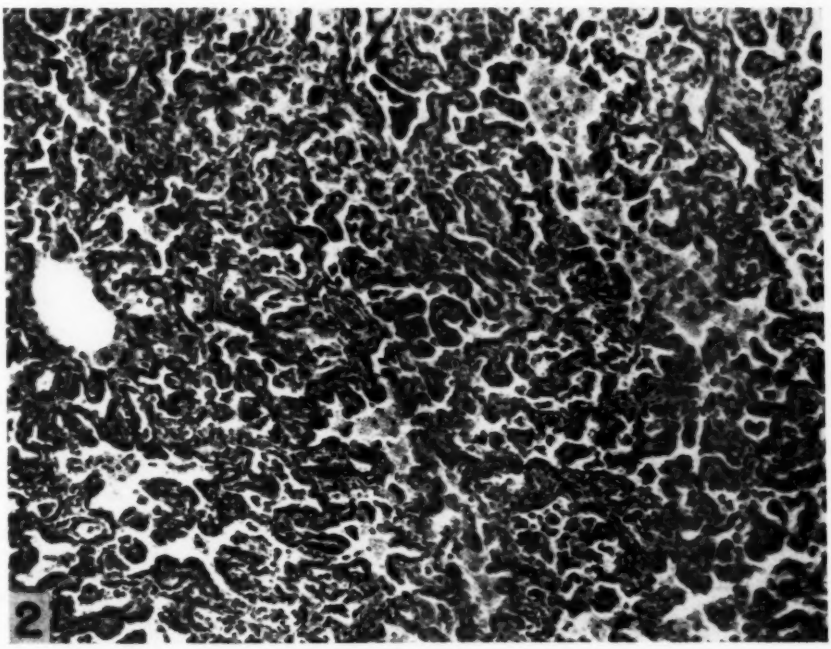

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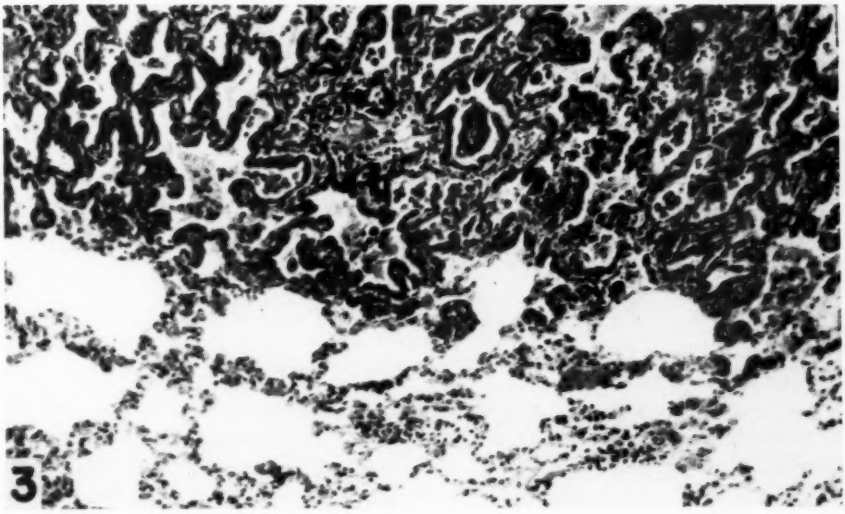
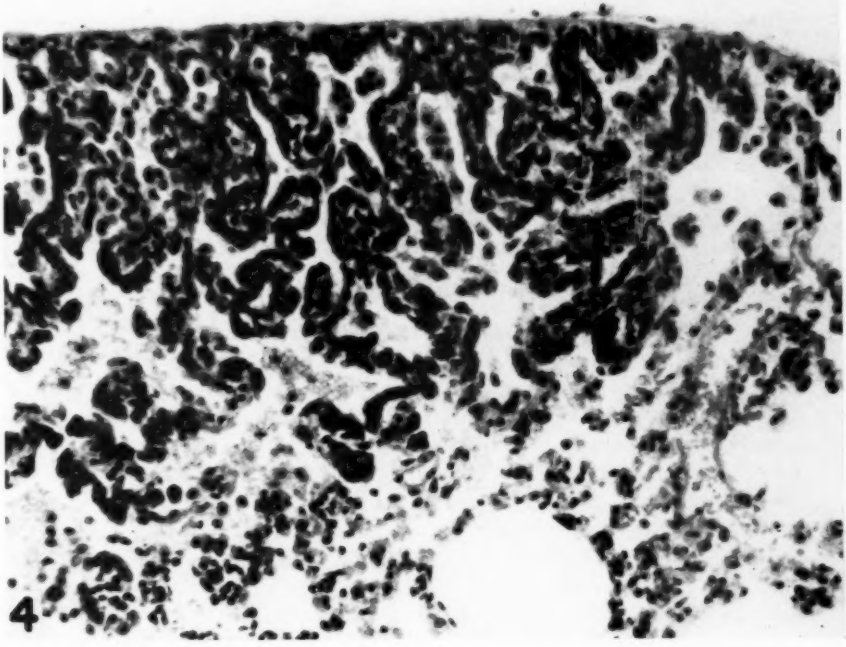
PLATE 73

FIGURE 3.—Junction of tumor with uninvolved lung. Hematoxylin and eosin. × 200

FIGURE 4.—Pleural surface of lung and partial lining of the junctional alveoli by tumor cells. Hematoxylin and eosin. × 275





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FACTORS MODIFYING THE EFFECT OF X-IRRADIATION ON REGRESSION OF A TRANSPLANTED LYMPHOSARCOMA [1]

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INTRODUCTION

The pronounced regression of a transplanted mouse lymphosarcoma caused by synergistic action of simultaneous body and tumor X-irradiation was described in a previous report (*1*). This paper deals with different factors modifying the irradiation effects on the tumor.

(1) The duration of the systemic factor, which plays a part in tumor regression when the body is also irradiated, was studied. Concomitantly the duration of the tumor damage, which makes it susceptible to the systemic factor, was investigated.

(2) To determine whether or not tumor regression is modified by starvation, the animals were starved prior to or following tumor irradiation.

(3) The effect of hydrogen peroxide on tumor regression was studied.

(4) A comparison of the modifications of regression caused by anoxia and hyperoxia was made.

(5) The modification caused by clamping off the blood supply to the tumor during local irradiation of the tumor was determined.

EXPERIMENTAL PROCEDURE AND RESULTS

In all experiments strain A male mice of the Bittner subline were used. They were inoculated subcutaneously with a transplantable lymphosarcoma at 3 to 4 months of age. The history of this tumor, designated L_1 has been described previously (*1*). Treatment with X-radiation was given 10 days following transplantation. The irradiation conditions were 186 kv. peak and 20 milliamps. Either a dose of 400 r or a dose of 1,300 r with differing dose rates was used. For the 400 r dose the beam was filtered with 0.12 mm. Cu and 0.55 mm. Al; the animals were placed 48.7 cm. from the target and irradiated for 5.2 minutes. The 1,300 r dose was given at 14.5 cm. from the target with a filter of 0.25 mm. Cu and 0.55 mm. Al and lasted 2 minutes. The tumor volume was determined with calipers.

[1] Received for publication September 24, 1951.

[2] National Institutes of Health, Public Health Service, Federal Security Agency.

When only the tumors were irradiated, each animal was placed in a 5-mm.-thick lead shield containing a slot through which the tumor was exteriorized. The dose to the body within the shield was measured with a 25 r Victoreen condenser ionization chamber with the mouse in place and was found to be less than 0.5 percent of the tumor dose.

When the tumors were shielded with 5 mm. lead shields during body irradiation the dose to the tumor was found to be 1 percent of the total dose.

Experiment 1

Eight groups of 20 animals each were treated with 400 r whole-body irradiation in the following manner: Half of the animals in each group were shielded, their tumors withdrawn from the shield, and irradiated locally. Then at given time intervals (24 hr., 6 hr., 4 hr., 1 hr., 5 min., and 2 min.) after the end of the tumor exposure, the bodies were irradiated while the tumors were shielded with 5 mm. of lead. In the remaining half of the animals the bodies were irradiated before the tumors. Fluorescein was injected intravenously into shaved mice enclosed in the shields. Examination under ultraviolet light showed fluorescein in the tumor, indicating that the blood supply of the tumor was not restricted.

In addition, one group of 10 animals was irradiated with 400 r whole-body irradiation with no shielding, and another group of 20 animals was given 400 r whole-body irradiation, during which the blood supply to the tumor was cut off with two steel rods 3 mm. in diameter taped tightly together. Again a check was made with fluorescein to show that blood did not enter the tumor while it was thus clamped off. Approximately 1 minute elapsed following removal of the clamp before the appearance of fluorescein in the tumor.

The criterion of effectiveness was taken as the average ratio between the minimum tumor volume and the volume at the beginning of treatment.

When the blood supply to the tumor was clamped off during total-body irradiation, tumor regression was negligible (table 1). Therefore, the

Table 1.—*Effect of irradiation of tumor with 400 r and body with 400 r at different time intervals*

Group A—Tumor irradiated first				Group B—Body irradiated first			
Time before body irrad.	Number of animals	Number unpalp.[1] tumors	$\frac{\text{Min. size}}{\text{Treat. size}} \times 100$	Time before tumor irrad.	Number of animals	Number unpalp. tumors	$\frac{\text{Min. size}}{\text{Treat. size}} \times 100$
24 hours	10	0	58±21	24 hours	10	0	74±19
6 hours	10	0	69±7	6 hours	10	0	75±30
4 hours	10	0	34±8	4 hours	10	0	43±12
2 hours	10	0	51±12	2 hours	8	0	26±15
1 hour	9	0	41±27	1 hour	7	0	24±19
5 minutes	10	0	33±15	5 minutes	6	0	30±24
5 minutes	10	0	33±13	5 minutes	10	0	58±22
2 minutes	10	0	13±15	2 minutes	10	0	22±22
0 minute	10	3	1.7±21	0[2]	20	0	92±0.5

[1] Tumor becoming unpalpable after treatment.
[2] Blood supply cut off.

primary effect of irradiation is instantaneous, and the presence in the tumor of this instantaneous factor is essential for a synergistic action between body and tumor.

When the tumor and body are irradiated at different times, the effectiveness of the treatment decreases as the interval between irradiation increases (table 1). Whether the body or the tumor is treated first does not seem to be of importance, as seen in text-figure 1. The decrease in effectiveness is rapid from 0 to 5 minutes; gradual from 5 minutes to 4 hours, and still more gradual from 4 hours on. The last point on the curve (text-fig. 1) was taken from the experiments in which the blood supply to the tumor was clamped off during irradiation. In this case, the body and the tumor can be considered as being irradiated at an infinite time apart, because the instantaneous factor in the body never enters the tumor. The nature of this time dependency is not clear. Whether it can be represented by a power function, a sum of exponentials, or some other function cannot be determined with the present data.

Experiment 2

Modification of the tumor irradiation effect by the metabolic state of the animal was tested in one group of 20 mice, deprived of food and water 20 hours before, and 4 hours following, tumor irradiation with 1,300 r. A second group of 20 mice deprived of food and water 4 hours before, and 20 hours following, tumor irradiation with 1,300 r served as controls. Tumor growth is not affected by starvation; in 10 mice deprived of food and water for 24 hours the rate of growth was comparable to that of 10 mice that were given food and water *ad libitum*.

While starvation following treatment did not affect regression, starvation before treatment increased tumor destruction as seen in table 2 and text-figure 2. The blood-glucose level, as determined by the Somogyi-

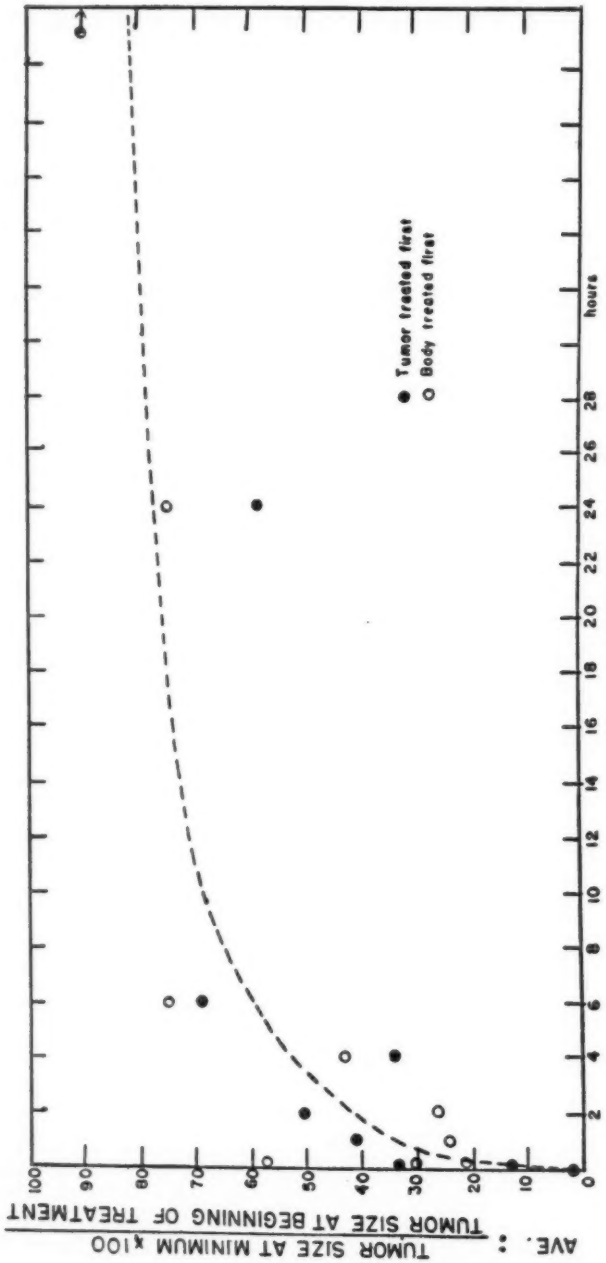
Table 2.—*The effect of starvation on regression of irradiated tumors*

Tumor X-ray dose	Treatment	Number of animals	Unpalp. tumors	$\frac{\text{Min. size}}{\text{Treat. size}} \times 100$	Level of significance compared with nonstarved animals [1]
1,300 r	None	20	5	28 ± 15	
1,300 r	Starved 20 hours prior to X-radiation.	20	5	6 ± 9	>99
1,300 r	Starved 20 hours following X-radiation.	19	0	29 ± 12	~20

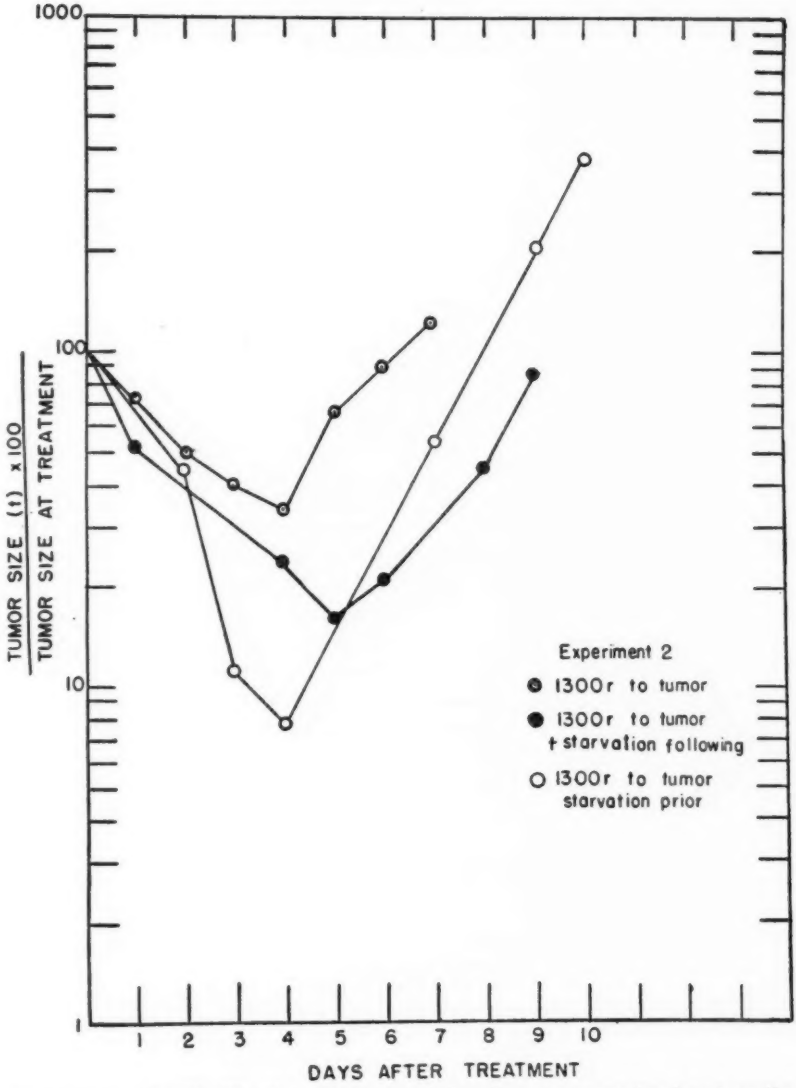
[1] P values as obtained from Fisher's "±" test.

Nelson method, of 10 animals starved in the manner described above was compared to that of 10 untreated mice. The blood-sugar level in the group given food and water *ad libitum* averaged 117 mg. percent, while the level of the starved animals averaged only 33.5 mg. percent.[3]

[3] We are indebted to Dr. S. Wollman for carrying out these determinations.



TEXT-FIGURE 1.—The time factor in synergism in body and tumor irradiation.



TEXT-FIGURE 2.—Modification of radiation-induced tumor regression by starvation.

EXPERIMENT 3

The effect on tumor regression of oxidizing agents acting directly on the blood or whole-animal system was studied in a group of 50 mice that were injected intravenously with 0.1 cc. of 0.66 percent H_2O_2, 10 to 20 seconds prior to local tumor irradiation with 1,300 r. A second group of 30 mice was injected with the same amount of H_2O_2 10 to 30 seconds following irradiation with 1,300 r to the tumor. The peroxide solution was titrated in acid with $KMnO_4$ to ascertain the concentration of peroxide.

In addition, as this oxidizing agent might substitute for total-body irradiation, two groups of 15 mice each were given 0.1 cc. of 0.66 percent H_2O_2 intravenously. The tumors of the mice of the first group were irradiated with 400 r 10 to 20 seconds following the injection. The second group was not irradiated. As shown previously (*1*) 400 r whole-body irradiation causes pronounced tumor regression.

Since the peroxide, in all probability, did not remain as peroxide in the body an appreciable length of time, any increase in tumor destruction caused by these injections was probably due to secondary reactions such as hyperoxia. The duration of these secondary reactions was studied as follows: Fifteen mice were injected with 0.1 cc. of the H_2O_2 solution and irradiated with 1,300 r to the tumor as previously. In another group of 15 mice the tumors were clamped off and 0.1 cc. of the peroxide solution was injected 5 minutes prior to irradiation. The clamp was removed immediately before irradiation of the tumor with 1,300 r.

A few of the peroxide-treated mice died, probably of pulmonary embolism, immediately following the injection. No significant difference was seen in any group of animals receiving like treatment. Therefore, all animals receiving the same treatment were lumped together in table 3 and text-figure 3. Tumor regression was greatly enhanced by the

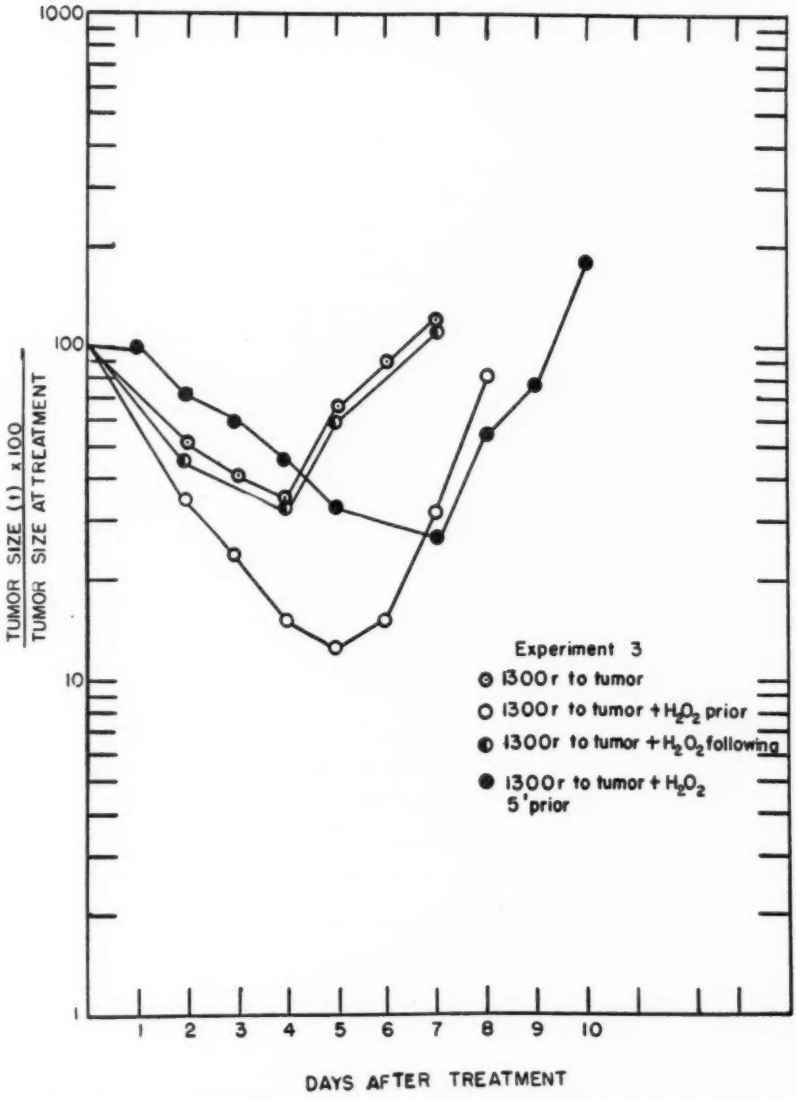
TABLE 3.—*Effect of H_2O_2 on regression of irradiated tumors*

Number animals surviving treatment	Local X-ray dose to tumor	H_2O_2 injection	$\frac{\text{Min. size}}{\text{Treat. size}} \times 100$	Level of significance compared with non-H_2O_2-treated animals
20	400 r [1]	None	88	
15	400 r	0.1 cc. 20″ prior	86±24	
15	None	0.1 cc	92±18	
30	1,300 r	None	27±32	
62	1,300 r	0.1 cc. 20″ prior	8.7±10.4	>99
26	1,300 r	0.1 cc. following	21.5±23.1	~50
19	1,300 r	0.1 cc. 5′ prior	18±22	~55

[1] Taken from previous experiments (*1*).

H_2O_2 injected 20 seconds prior to irradiation, but not affected by H_2O_2 injection following irradiation as seen in table 3 and text-figure 3.

The effect, however, is not great enough to be comparable with 400 r whole-body irradiation, as no modification of the effect of 400 r radiation to the tumor is apparent although more peroxide was injected than



TEXT-FIGURE 3.—Modification of radiation-induced tumor regression by H_2O_2 administration.

could be formed by 400 r in the body tissues. Furthermore, peroxide alone does not appear to effect tumor regression. It might, however, slow down tumor growth, since in 4 out of the 15 mice treated with peroxide alone the tumor regressed slightly the day after H_2O_2 injection.

Experiment 4

Two groups of 20 mice each were placed in the lead shields, and their tumors withdrawn and locally irradiated with 1,300 r. In one group a mixture of 95-percent oxygen and 5-percent carbon dioxide was fed into the air intake at the front of the shields. The mixture flowed through the shields 2 minutes preceding radiation and during radiation at the rate of 1 liter per minute. The second group was given a mixture of about 92-percent nitrogen and 8-percent oxygen at the same rate and for the same period as those above.

Two animals died in the nitrogen atmosphere during exposure. No evidence was obtained that the first group was hyperoxic and the second group anoxic, although attempts to detect a change in oxyhemoglobin with change in atmosphere were made with an oxymeter. Nevertheless, a 10-fold difference occurred in the tumor regression of the two groups. Increased oxygen increased the regression while decreased oxygen decreased tumor destruction. (See table 4 and text-fig. 4.)

Table 4.—*Effect of composition of inspired atmosphere on regression of irradiated tumors*

Tumor X-ray dose	Atmosphere at radiation	Number of animals	Number unpalp. tumors	$\frac{\text{Min. size}}{\text{Treat. size}} \times 100$	Level of significance compared with animals in air
1,300 r	95% O_2+5% CO_2	20	6	3.4± 5.0	>99
1,300 r	92% N_2+8% O_2	18	2	40.9±12.9	>99
1,300 r	Air	20	5	28±15	

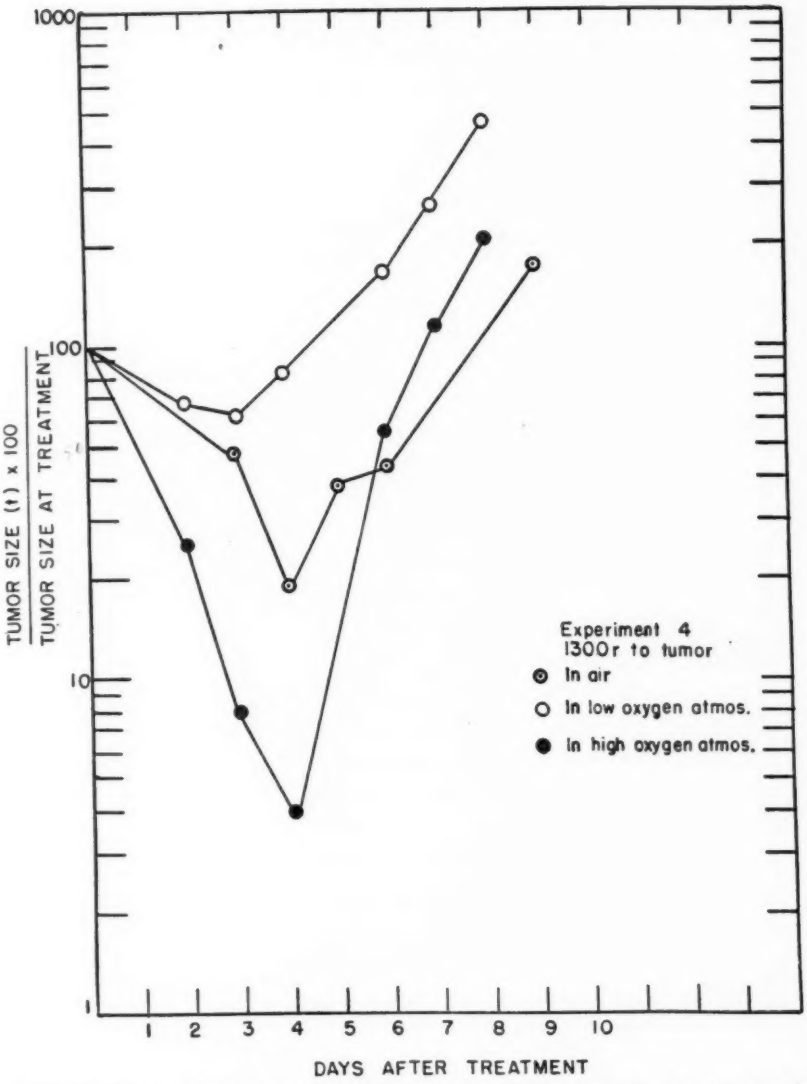
Experiment 5

No tumor regression occurred with 400 r whole-body irradiation when the blood supply to the tumor was cut off. The question arose whether or not clamping off the blood supply to the tumor decreased tumor destruction following local tumor irradiation. Therefore, a group of 28 animals was given 1,300 r X-irradiation to the tumor with the blood supply clamped off during irradiation. Another group of 29 mice was given 1,300 r to the tumor without vascular compression.

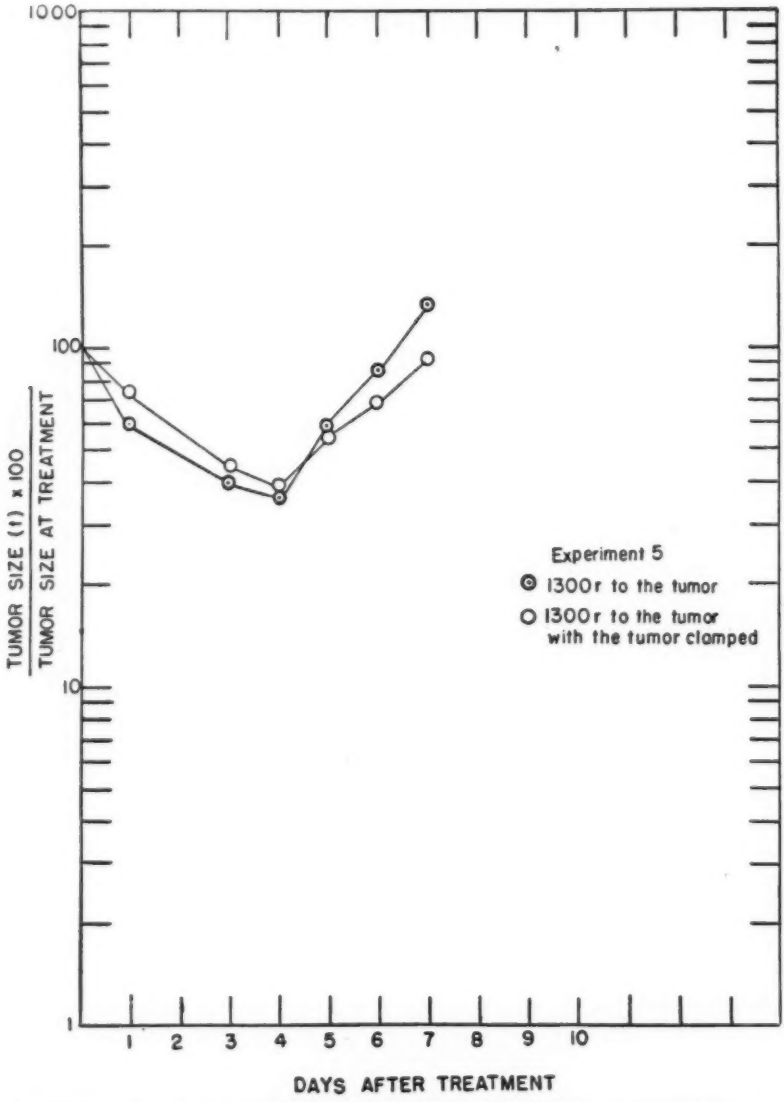
The clamping off of the tumor for this short period does not modify the effect of tumor irradiation as seen in table 5 and text-figure 5.

Table 5.—*Effect of clamping off blood supply to the tumor during irradiation*

Number animals surviving treatment	X-ray dose to tumor	Other treatment	Number unpalp. tumors	$\frac{\text{Min. size}}{\text{Treat. size}} \times 100$
28	1,300 r	None	5	24.8±24.4
29	1,300 r	Tumor clamped	3	24.3±24.0



TEXT-FIGURE 4.—Modification of radiation-induced tumor regression by anoxia and hyperoxia.



TEXT-FIGURE 5.—Lack of modification of radiation-induced tumor regression by clamping off the blood supply to the tumor.

DISCUSSION

While measurement of the tumors with calipers is a crude method of determination of radiation damage, any inaccuracy of the measurements is small in comparison to the large biologic variations encountered. Perhaps differences in the amount of surrounding tissue in local tumor irradiation account for part of this variation. Therefore, only large differences in effects can be noted. When the experiment indicates a negative response, *e. g.*, the failure of peroxide injected 5 minutes before irradiation to enhance the radiation effect, it only indicates that no large response has taken place.

Another difficulty encountered in these experiments that led to an additional inaccuracy was the instability of the peroxide solution at room temperature and the formation of oxygen in the tail vein at the site of injection. Most of the embolisms, we believe, were produced by gas formed in the syringe when the blood backed into the needle before injection. In spite of the titrations to insure constant amounts of peroxide in the solution, constant amounts were not always delivered to the animals.

The results of Experiment 1 indicate, not only that the effect of irradiation is instantaneous, but also that the instantaneous factors must be present in the tumor as well as in the body to produce the synergism. In addition, free exchange of these factors in tumor and body is necessary, for when the tumor is clamped off during total-body irradiation negligible tumor regression occurs, even though the clamps are removed within 15 seconds after the end of the treatment.

Some synergism does occur, however, if the tumor and body are irradiated at different times. The extent of this synergism is independent of the order in which the body and tumor are irradiated but dependent on the time between treatments. The conclusion can be drawn that the instantaneous factor is the same in the body and in the tumor. It has its maximum effect in simultaneous irradiation of body and tumors. It apparently gives rise to a series of unknown reactions that disappear in time. The lack of effect on the tumor when its blood supply was cut off during total-body irradiation is not in contradiction to the experiments in which the time factor was studied. Apparently free circulation is necessary at all times for the action of the synergistic factors.

Anoxia in the tumor during the clamping-off period also does not explain the lack of tumor regression following total-body irradiation, for when the tumor was clamped off during local irradiation to 1,300 r tumor regression was not affected.

Furthermore, another point is cleared up: that of the effect on tumor growth of the manifestations of radiation injury such as leukopenia, decreased food intake, *etc.* These manifestations are present whether the animals are irradiated with 400 r to the body while the tumor is shielded, or 400 r to the whole body while the tumor is clamped off, or irradiated with 400 r to the total body. Yet only in the latter case does pronounced regression occur. Hence, it can be concluded that enhance-

ment of tumor destruction is not caused by such effects of radiation injury.

While the starvation experiment shows that the metabolic state of the animal at the time of tumor irradiation is important and indicates that the irradiation synergism may be metabolic in nature, it is difficult to evaluate. Certainly, the starvation in itself does not affect tumor growth as seen in the data of the animals starved following irradiation or in starved nonirradiated animals. On the other hand, in acutely starved animals the blood-sugar content is very low, and it is known that glucose protects animals from radiation death when given prior to irradiation (*2*). Lack of glucose can conceivably increase radiation damage. Other effects of starvation of the duration used here may be adrenal stimulation, which not only produces protein depletion but also the depletion of muscle and liver glycogen (*3*) and involution of lymphoid tissues.

Because hydrogen peroxide is formed by irradiation in water, and consequently also in tissue cultures and other water-containing media, it probably plays a role in the irradiation effects of animals (*4*). The increased tumor regression in the mice treated with peroxide prior to irradiation is, therefore, not surprising. The lack of effect in the mice treated with peroxide following irradiation was expected from the time study (experiment 1). While the peroxide most probably does not remain in the mice as peroxide at the time of the irradiation, it possibly did start a chain of events of short duration that were active at the time of irradiation. The short duration of such reaction was shown in the experiment in which a delay of 5 minutes between injection and irradiation did not produce an enhanced tumor regression. With 400 r no apparent synergism occurred between peroxide and tumor irradiation. Secondary products of H_2O_2 may not be enough to affect the small reaction of the tumor to 400 r, but these secondary products are apparently great enough to affect the tumor that is damaged by 1,300 r.

The increased regression of the tumor treated while the mice were in the higher-oxygen atmosphere and the decreased regression of the tumor treated while the mice were in a low-oxygen atmosphere agrees with observations of many others (*2*). Thoday and Read (*5*) showed that treatment of the *Vicia faba* root with nitrogen decreased its sensitivity to X-radiation, while treatment with oxygen increased its sensitivity. Dowdy *et al.* (*6*) likewise found that an atmosphere of low-oxygen content protected rats and mice from irradiation death.

The difference in reactions may be caused by a difference in metabolic rate, but more likely it is caused by a difference in the amount of oxygen dissolved in the plasma, and tissue fluids.

SUMMARY

A synergistic effect on regression of a transplanted mouse lymphosarcoma occurred after simultaneous systemic and local tumor irradiation and was absent when the blood supply of the tumor was clamped off during systemic irradiation. The synergistic effect disappeared rapidly when the time interval between systemic and local tumor irradiation was increased from 0 minutes to 4 hours.

Starving the animals for 20 hours before and during local irradiation of the tumor was found to increase tumor regression, while starvation following irradiation had no effect.

Hydrogen peroxide administered intravenously to the tumor-bearing animals immediately before tumor irradiation increased the effect on tumor regression in comparison to noninjected irradiated controls, but hydrogen peroxide administered after irradiation did not. Hydrogen peroxide injected 5 minutes before irradiation did not significantly increase tumor damage.

Irradiation of the tumors of mice that were kept in a high-oxygen atmosphere produced a greater regression, and irradiation of the tumors of mice in a low-oxygen atmosphere produced less regression than irradiation of the tumor of mice breathing air.

Clamping off the blood supply to the tumor during local irradiation of the tumor had no effect on tumor regression.

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MORTALITY OF MICE AFTER TOTAL-BODY IRRADIATION AS INFLUENCED BY ALTERATIONS IN TOTAL DOSE, FRACTIONATION, AND PERIODICITY OF TREATMENT [1] [2]

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INTRODUCTION

Studies of lethal dose and survival time for total-body exposure of mice to roentgen rays have been reported (*1*, *2*); comparable studies have also been made with other species (*3*). More recently, the effect of dose fractionation on radiation mortality of mice has received attention (*4*, *5*). Significant gaps have remained, however, in the available data on radiation mortality, particularly with respect to the quantitative effects of dose fractionation and of intermittent, periodic irradiation.

The data reported herein are the by-product of an experimental study of the effect of alterations in total dose, fractionation, and periodicity of irradiation on the development of lymphoid tumors in mice. The lymphoid-tumor dose-response data will be reported separately. It was known, however, that initial mortality (defined for this study as occurring within 60 days after the start of irradiation) could be anticipated at many of the dose levels to be employed. The experimental design was therefore planned to yield data concerning both sequelae of irradiation.[3]

EXPERIMENTAL PROCEDURE

A total of 1,753 C57 black mice of both sexes, aged 33 ± 3 days at the start of irradiation, was used.[4] Whole litters of 3–8 mice were weaned at 25–28 days of age and assigned (using a table of random numbers) to one

[1] Received for publication September 25, 1951.

[2] This investigation was supported by a grant from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service. Presented in part at the Symposium on Radiobiology, 117th annual meeting, American Association for the Advancement of Science, Cleveland, December 29–30, 1950.

[3] Grateful acknowledgement is made to Professor Albert H. Bowker, of the Department of Statistics, Stanford University, and to Mr. Jerome Cornfield and Mr. Nathan Mantel, statisticians of the National Cancer Institute, for generous assistance and advice in the planning of this experiment, and to Professor Bowker, Dr. J. Yerushalmy, and Mr. William Taylor, statisticians in the School of Public Health, University of California at Berkeley, for guidance in the application of the probit method to the experimental data.

[4] Breeder animals of the Yale subline of this strain were originally obtained through the generosity of Dr. Leonell C. Strong, Department of Anatomy, Yale University. All subsequent generations have been obtained by pedigreed brother-to-sister matings in our laboratory.

of 35 treatment groups. Litters that appeared unusually small or included abnormal members were discarded. Individual litters were irradiated in shallow, cylindrical, perforated lucite boxes, 3.5 cm. high and 25 cm. in diameter, suspended in air in the X-ray beam.[5]

Physical factors of irradiation for the original X-ray unit were: 120 KVP, 8 ma., 0.25 mm. Cu and 1.0 mm. Al added filter, 30 cm. target-to-mouse distance, H. V. L. 0.33 Cu, output 25 r per min. This apparatus ceased to operate and was replaced with another operating at 120 KVP, 9 ma., 0.25 mm. Cu and 1.0 mm. Al added filter, 30 cm. target-to-mouse distance, H. V. L. 0.37 mm. Cu, output 30–32 r per min. During the installation of the new unit, some treatments were given at 140 KVP, 20 ma., no added filter, 40 cm. distance, H. V. L. 0.26 mm. Cu, 58.5 r per min. No studies were conducted to determine the magnitude of variation in response due to these differences in physical factors.

A graded sequence of total doses was employed, each dose being the product of $\sqrt[4]{2}$ and the next lower dose. In Series 1, each dose was given as a single exposure. In Series 2, 3, and 4, the total doses were divided into 2, 4, or 8 equal fractions, respectively, given on consecutive days. Finally, at three selected levels of total dose, four equally fractionated treatments were given in Series 5, 6, and 7 at intervals of 4, 8, and 16 days, respectively. The dose levels of Series 1–4 were staggered somewhat to allow for anticipated decreases in response with increasing fractionation. Larger numbers of mice were assigned to high-dose groups in the hope of obtaining enough survivors to provide valid lymphoid-tumor data (table 1).

TABLE 1.—*Experimental design*

Series	Number of treatments	Interval between treatments (days)	Total dose (roentgens)								
			283	336	400	475	566	673	800	951	1,131
1	1	0	[1] 27	31	42	42	84	----	----	----	----
2	2	1	31	31	29	39	73	81	63	----	----
3	4	1	----	39	42	36	56	70	92	60	----
4	8	1	----	----	33	32	36	43	55	50	56
5	4	4	----	----	----	51	----	49	----	56	----
6	4	8	----	----	----	54	----	48	----	60	----
7	4	16	----	----	----	51	----	53	----	58	----

[1] Initial number of mice treated.

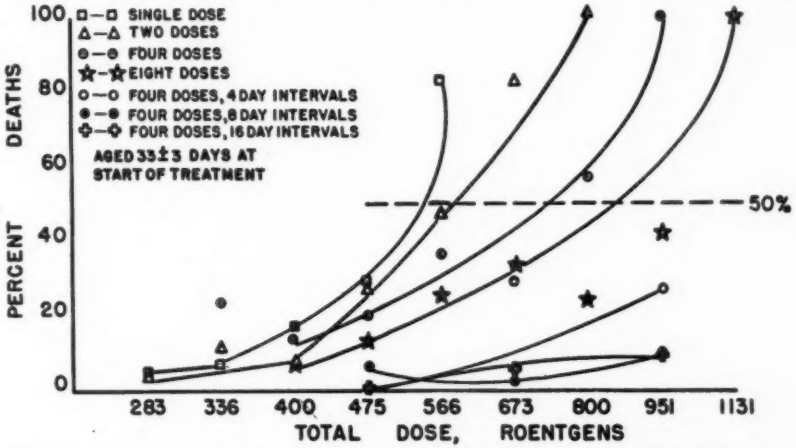
Throughout the experiment, animals were identically caged in wooden boxes with free access to Purina laboratory chow and water. Essentially uniform room temperatures were maintained, and no significant laboratory infections occurred during the experiment. Cages were checked daily for dead animals, but routine autopsies were not performed on mice dying within the first 30 days after irradiation. Nine animals died of extraneous causes, such as trauma during routine handling; since the number of such

[5] In another experiment the addition of a scattering medium (rice) did not affect dose significantly, despite eported observations to the contrary.

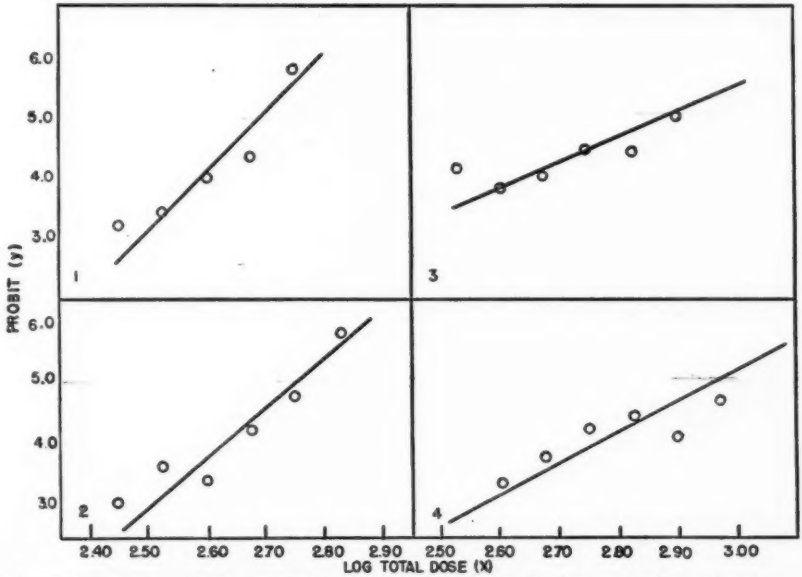
deaths was only 0.5 percent of the entire population, however, no correction for control deaths was necessary on subsequent analysis of the data by the probit method (*6*).

RESULTS

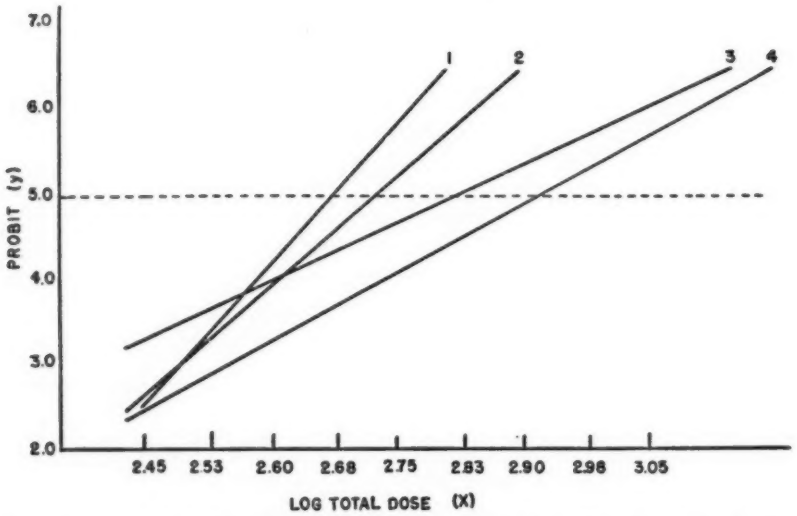
Mortality by groups is given in table 2, and text-figure 1. Probits have been determined from data of Series 1–4, and the calculated probit regres-



TEXT-FIGURE 1.—Quantitative data on radiation mortality in strain C57 black mice.



TEXT-FIGURE 2.—Probit regression lines for postirradiation mortality of mice.
Series 1=Single treatment.
Series 2=Two equal daily treatments.
Series 3=Four equal daily treatments.
Series 4=Eight equal daily treatments.



TEXT-FIGURE 3.—Comparative probit regression lines for mortality of mice after fractionated total-body irradiation.
Series 1=Single treatment.
Series 2=Two equal daily treatments.
Series 3=Four equal daily treatments.
Series 4=Eight equal daily treatments.

sion lines for each series, together with the experimental points, are plotted in text-figure 2. For ease of comparison, these regression lines are plotted on the same graph in text-figure 3, with the 50-percent mortality level ($y=5.0$) indicated as a horizontal interrupted line. LD–50 values are the anti-logarithms of the log dose, x_m, for $y=5.0$ along each regression line. The LD-50 (and standard error) for single whole-body irradiation was 486 r$\pm$18, increasing with fractionation to 542 r$\pm$22, 678 r$\pm$71, and 850 r$\pm$80 for Series 2, 3, and 4, respectively.

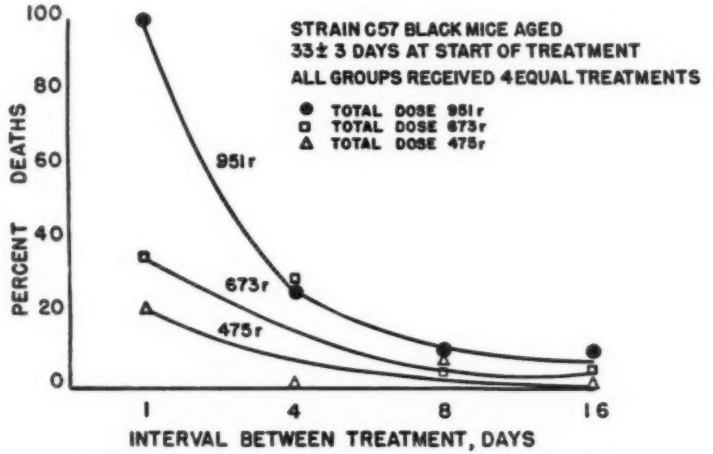
At all 3-dose levels employed, mortality was much lower after the intermittent periodic irradiation of Series 5–7, and did not approach 50 percent in any case. For this reason, calculation of the probit regression line for these three series was unreliable; additional data at higher dose levels will be necessary before valid LD-50 values can be assessed for irradiation administered in this manner. Nevertheless, the present data clearly reveal that the interposition of an interval of 4 or more days between successive fractionated exposures, at relatively high levels of total dose, results in a profound drop in mortality (text-fig. 4), presumably because of recovery between exposures.

The peak frequency of death, as indicated by tabulated number of deaths per day (text-fig. 5), occurred earlier as total dose and final mortality increased in the various groups. Although observations were

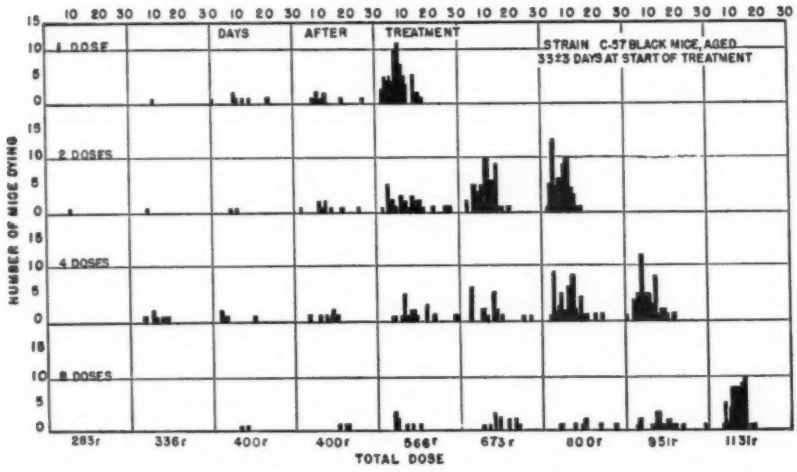
TABLE 2

Series	Number of treatments	Interval between treatments (days)		Total dose (roentgens)								
				283	336	400	475	566	673	800	951	1,131
1	1	0	Number of mice [1]	27	31	42	42	84				
			Radiation deaths [2]	1	2	7	12	70				
			Mortality, percent	**4**	**6**	**17**	**29**	**83**				
			Mean survival,[3] days	34	33	11	17	10				
2	2	1	Number of mice	31	31	29	39	71	81	63		
			Radiation deaths	1	3	2	10	33	67	63		
			Mortality, percent	**3**	**10**	**7**	**26**	**46**	**83**	**100**		
			Mean survival, days	9	36	9	18	13	12	8		
3	4	1	Number of mice		39	42	35	56	70	91	60	
			Radiation deaths		9	6	7	20	23	52	60	
			Mortality, percent		**23**	**14**	**20**	**36**	**33**	**57**	**100**	
			Mean survival, days		17	14	13	15	12	10	9	
4	8	1	Number of mice			33	32	36	42	54	50	56
			Radiation deaths			2	4	9	14	12	21	56
			Mortality, percent			**6**	**13**	**25**	**33**	**22**	**42**	**100**
			Mean survival, days			13	23	13	16	23	15	11
5	4	4	Number of mice				51		49		55	
			Radiation deaths				0		14		15	
			Mortality, percent				**0**		**29**		**27**	
			Mean survival, days						20		16	
6	4	8	Number of mice				54		48		60	
			Radiation deaths				4		1		6	
			Mortality, percent				**7**		**2**		**10**	
			Mean survival, days				29		28		23	
7	4	16	Number of mice				51		52		57	
			Radiation deaths				**0**		2		5	
			Mortality, percent				**0**		**4**		**9**	
			Mean survival, days						17		33	

[1] Deaths from extraneous causes were negligible and have been omitted.
[2] Deaths within 60 days after start of irradiation.
[3] Days after first X-ray treatment.



TEXT-FIGURE 4.—Effect of periodicity on radiation mortality.



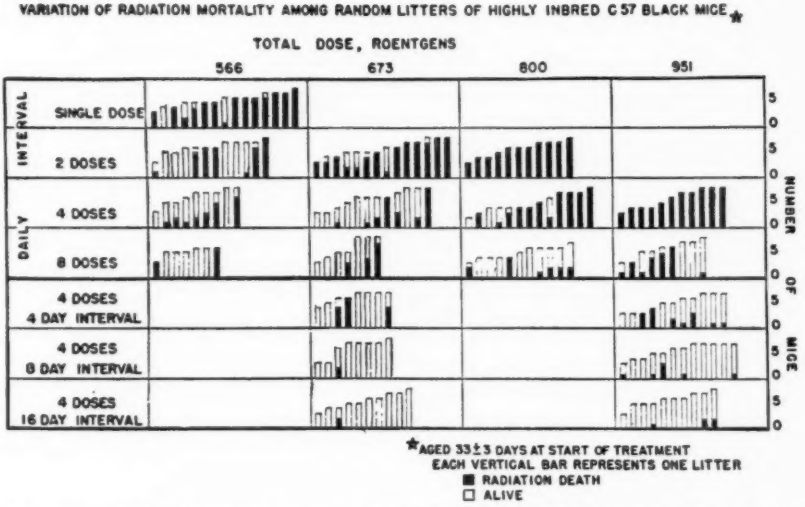
TEXT-FIGURE 5.—Relation of survival time to fractionation of whole body irradiation (daily fraction).

continued through 60 days, virtually all the deaths in Series 1–4 occurred within 30 days after irradiation. Mean survival times have been calculated for the animals dying in each group (table 2), but are not reliable for the low-dose levels in any series because too few animals succumbed in these groups.

There was no sex difference in mortality or survival time (table 3). Of the determinate total of 1,744 mice irradiated, 874 were females and 870 males. There were 613 postirradiation deaths (35.15 percent), of

which 307 (35.13 percent) occurred in females and 306 (35.17 percent) in males. This result is in agreement with previous experience in this laboratory with C57 black mice (7), but does not exclude the possibility of a sex difference in other strains.

A striking variation in response from litter to litter was noted in many treatment groups, in some instances with survival of entire large litters and death of others exposed to the same dose (text-fig. 6). Statistical



TEXT-FIGURE 6.—Mortality among random litters of highly inbred C57 black mice.

analysis of this data in relation to the incidence of death for each group yielded several values of χ^2 far exceeding that associated with a probability of 0.01, as seen in table 4.[6]

Interpretation of this result is not clear. No correlation could be detected between the degree of susceptibility or resistance of a given litter and that of other litters from the same lineage, suggesting that this response is not genetically determined. Analyses of the relation between

TABLE 3.—*Postirradiation mortality of sexes*

Number of mice treated	♀	♂	Total
	874	870	1,744
Radiation deaths	307	306	613
Mortality, percent	35.13	35.17	35.15
Mean survival time (days)	12.7	12.4	12.68

[6] We are deeply indebted to Mr. William F. Taylor of the School of Public Health, University of California at Berkeley, for this statistical analysis.

TABLE 4.—*Variation in Litter Response: values of the chance, p. of survival: χ^2 as used in the test for homogeneity; number, d. f., of degrees of freedom and χ^2 corresponding to 0.01 significance level.*

	Dosage level							
	283	336	400	475	566	673	800	951
Single dose:								
p	1/27	2/31	7/42	12/42	70/84	------	------	------
χ^2	8. 3	6. 1	22. 8	28. 9	**57. 4**	------	------	------
d. f	5	6	8	9	14	------	------	------
0.01 critical level	15. 09	16. 81	20. 09	21. 67	29. 14	------	------	------
2 daily doses:								
p	1/31	3/31	2/29	10/39	33/71	67/81	------	------
χ^2	7. 0	4. 8	13. 4	17. 7	**60. 0**	**41. 4**	------	------
d. f	6	5	4	5	11	13	------	------
0.01 critical level	16. 81	15. 09	13. 28	15. 09	24. 72	24. 69	------	------
4 daily doses:								
p	------	9/39	6/42	7/35	20/56	23/70	------	------
χ^2	------	13. 6	22. 1	**37. 2**	17. 2	**42. 2**	------	------
d. f	------	6	7	7	8	11	------	------
0.01 critical level	------	16. 81	18. 47	18. 47	20. 09	24. 72	------	------
8 daily doses:								
p	------	------	2/33	4/32	9/36	14/42	12/54	21/50
χ^2	------	------	7. 9	8. 2	**36. 0**	**22. 0**	24. 0	**33. 7**
d. f	------	------	5	5	6	6	10	8
0.01 critical level	------	------	15. 09	15. 09	16. 81	16. 81	23. 21	20. 09

mortality and (a) initial number of mice per litter (table 5) and (b) sequence of litter in maternal obstetrical history (table 6), revealed no consistent trends; the variations are readily accounted for by differences in litter distribution among high- and low-dosage groups. The litters were derived from six different generations, but this factor likewise appeared to exert little or no influence on susceptibility (table 7).

TABLE 5.—*Mortality and initial number of mice per litter*

Initial number of mice in litter	3	4	5	6	7	8	Total
Number of mice treated	143	231	317	363	426	264	1, 744
Radiation deaths	56	83	71	124	160	119	613
Mortality (percent)	**39**	**36**	**22**	**34**	**38**	**45**	**35**

TABLE 6.—*Litter sequence and mortality*

Obstetrical sequence of litter	1	2	3	4	5	6	Total
Number of mice treated	477	408	328	345	160	26	1, 744
Radiation deaths	178	167	118	102	38	10	613
Mortality (percent)	**36**	**41**	**36**	**30**	**24**	**38**	**35**

TABLE 7.—*Litter generation and mortality*

Generation of litter	1	2	3	4	5	6	Total
Number of mice treated	20	297	674	546	194	13	1, 744
Radiation deaths	7	120	232	190	54	10	613
Mortality (percent)	**29**	**40**	**35**	**35**	**28**	**77**	**35**

A "cage" effect has been postulated to explain the occasionally extreme variation in litter response; this explanation seems unlikely, however, in relation to the laboratory conditions that existed during the experiment. Another tentative explanation is based upon the hypothesis that susceptibility is not homogeneously distributed through the entire mouse population, but rather that litters tend to group into "resistant" and "susceptible" categories, within which individual mortality is then governed by chance. In any case, the magnitude of the litter effect observed in some instances provides additional evidence to support the experimental practice of litter-mate pairing wherever possible, together with the use of adequate numbers of animals, in the design of biologic experiments.

DISCUSSION

The results in Series 1–4 are in agreement with earlier evidence (*1*, *3*), that the lethality of systemic X-irradiation increases sigmoidally with total dose, and that the effect of a given dose decreases when that dose is given in multiple fractions rather than at one time (*5*). No evidence of a plateau in the range from 200 to 600 r, as described by Quastler (*2*), was found. There is a striking decrease in lethality as the time interval between fractional doses is increased. Unfortunately, the data of Series 5–7 do not provide a basis for determination of the LD–50; additional studies on periodic irradiation will be necessary before the magnitude of this decrease in effect can be expressed in terms of the increase in LD–50, as is now possible for simple daily fractionation.

MATHEMATICAL CONSIDERATIONS[7]

The increase in LD–50 noted in Series 1, 2, 3, and 4, where the total dose was given in 1, 2, 4, or 8 (respectively) equally fractionated doses, suggests the possibility that the dependence of the LD–50 on the degree of fractionation may be expressible in some simple mathematical form.

Let m_i stand for the logarithm of the observed LD–50 in series i ($i=1,2,3,4$) and f_i be the number of equal fractions in the series i ($f_1=1$; $f_2=2$; $f_3=4$; $f_4=8$). Let μ_i denote the "true" value of the logarithm of the LD–50.

Suppose that the nature of the mechanism causing fractionation to increase the LD–50 can be expressed by the equation:

$$\mu_i = a + R \log (f_i) \qquad i=1,2,3,4$$

We wish to

(1) Estimate the parameters a and R by $\hat{a}$ and $\hat{R}$, respectively, and find the variance of these estimates.

(2) Test whether the data fit the above model sufficiently well.

Using the method of maximum likelihood (which for this problem is

[7] By L. E. Moses and Professor Albert H. Bowker, Department of Statistics, Stanford University, Stanford, Calif.

identical with the method of least squares) we arrive at the following values:

$$\hat{R}'=0.2115 \qquad \text{Var. } \hat{R}'=0.00161$$
$$\hat{a}=2.6850 \qquad \text{Var. } \hat{a}=0.000215$$

and the estimates of μ_i using these estimates of the parameters are:

$$\hat{m}_1=2.685$$
$$\hat{m}_2=2.749$$
$$\hat{m}_3=2.812$$
$$\hat{m}_4=2.876$$

These may be compared with the observed values of the logarithm of the LD–50.

$$m_1=2.687 \qquad s_1=0.0171$$
$$m_2=2.734 \qquad s_2=0.0177$$
$$m_3=2.831 \qquad s_3=0.0481$$
$$m_4=2.929 \qquad s_4=0.0409$$

To assess how well the data fit the model a χ^2 test of the form:

$$\sum_{i=1}^{4}\frac{(m_i-\hat{m}_i)^2}{\sigma_i^2}=\chi_3^2$$

is appropriate.

The value of χ^2 obtained is 2.59, which lies very near the 50$^{\text{th}}$ percentile of the χ^2 distribution with three degrees of freedom, indicating a good fit of the data to the model chosen. It is worth while noting that the model used can also be written in the form:

$$LD_{50}^{(f)}=LD_{50}^{(1)}\,(f)^R$$

Where: $LD_{50}^{(1)}$ is the dose causing 50 percent mortality, the whole dose being given in one exposure;

f is the number of equal parts into which a total dose is fractionated;

$LD_{50}^{(f)}$ is the dose (fractionated into f equal parts) causing 50 percent mortality.

For the data of this investigation, the estimate of the true relation of this form is:

$$LD_{50}^{(f)}=484.2\,(f)^{0.2115}$$

denoting that $LD_{50}^{(f)}$ increases roughly as the 5$^{\text{th}}$ root of the degree of fractionation, over the range of fractionation used in this study.

This model permits a series of predictions concerning the effect of fractionation that can be submitted to the test of further experimentation. It will be necessary to determine experimentally whether the effects described are due to fractionation of dose *per se* or to the associated extension of time during which radiation is delivered. Other experimental evidence (*8*), indicates that response is probably dependent on both dose per fractional exposure and time interval between exposures. It

would also be of interest to learn whether the same or a similar model will fit data obtained for fractionated treatment at some other level of effect; *e. g.*, LD-25 or LD-75.

SUMMARY

The lethality of total-body X-irradiation was studied in 1,753 strain C57 black mice of both sexes, 1 month old at the start of treatment, randomized by litters into 35 groups, that received a graded sequence of total doses in single exposures or 2, 4, or 8 equal fractions at intervals of, 1, 4, 8, or 16 days. There was no sex difference in mortality. Analysis of the data by the probit method yielded regression lines with LD-50 values of 486 r$\pm$18 for single exposures, increasing to 542 r$\pm$22, 678 r$\pm$71, and 850 r$\pm$80 for 2, 4, or 8 daily fractionated exposures, respectively. A pronounced decrease in lethal effect was noted when the interval between fractionated exposures was increased to 4, 8, or 16 days, but the data do not permit accurate estimation of the LD-50 levels under these conditions. A mathematical model is presented that fits the present data for daily fractionation and provides a tentative framework for additional dose-response experiments.

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981784—52——8

ACUTE NECROTIZING RENAL PAPILLITIS EXPERIMENTALLY PRODUCED IN RATS FED MONO-N-METHYLANILINE [1]

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Many attempts have been made to produce hepatic tumors by the oral administration of products that may result from the possible breakdown of *p*-dimethylaminoazobenzene with little success. For instance, results of feeding aniline have previously been reported whereby prolonged feeding resulted in 20 percent hepatic tumors (*1*). The present paper reports pathologic changes observed after the oral administration of mono-N-methylaniline given as the hydrochloride in an attempt to produce hepatic tumors. Although no hepatic tumors developed, an interesting and unusual renal lesion was observed in many of the treated animals.

METHODS AND OBSERVATIONS

Twenty female and 20 male Osborne-Mendel rats weighing approximately 70–80 gm. were housed individually in suspended cages and fed the standard basal diet to which was added 0.06 percent mono-N-methylaniline hydrochloride.[4] In addition, each animal was given a supplement of 400 mg. of dried brewer's yeast and 0.5 percent L-cystine daily and 0.5 ml. of cod-liver oil weekly. The animals were given the diet and tap water *ad libitum*. The weights were recorded twice a week, and the daily amount of food intake was calculated and recorded.

The females averaged 479 days on this experimental diet, with an average daily food intake of 8.8 gm. per animal. The males showed a slightly higher daily food intake of 9.5 gm. and averaged 562 days on the diet. All animals appeared to do well, as evidenced by the food intake and also by an average daily weight gain of from 0.69 gm. to 0.79 gm., until a few days prior to killing the animals, when the food intake was markedly reduced and the animals showed signs of lethargy and malaise.

Of the 40 rats in the experiment, 7 died and 30 were killed at various

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[4] Diet (G 6 C MAH) consisted of 6 percent casein (crude), 50.44 percent starch, 24 percent Crisco, 15 percent sucrose, 4 percent Osborne-Mendel salts, 0.06 percent mono-N-methylaniline hydrochloride (MAH).

intervals between 272 and 758 days because they appeared to be sick. The remaining 3 apparently healthy rats were killed at the termination of the experiment.

Gross examination.—On gross examination it was noted that there were large deposits of adipose tissue throughout the body of all animals. The liver was pale or yellowish-brown in color, and sometimes hemorrhagic foci were observed. The spleen, as well as the kidneys, appeared moderately congested in the majority of animals. No tumors were found in the liver or in any other organs or tissues.

Microscopic findings.—The liver of most of the animals showed a remarkable fatty change, and in some animals early cirrhosis was present. Occasionally vascular thrombosis and several infarcts or focal necroses were found in the liver (fig. 1). The spleen showed numerous brown pigmented macrophages in the red pulp (hemosiderosis), and there were occasional small infarcts (fig. 2).

The most striking pathologic lesion was found in the papilla of the kidney. This lesion was primarily vascular and consisted of venous congestion and hyaline thrombosis in the smaller vessels. A deposit of calcium-like material that stained deep blue with hematoxylin and black with the von Kossa's silver impregnation was sometimes present in the thrombosed vessels and less frequent in the collecting tubules (fig. 3). The striking lesions were confined to the tip of the papilla, where necrosis with focal hemorrhage was frequent. The epithelium covering the papilla, as well as the epithelium at the orifice of the collecting tubules, in many cases had undergone necrosis, so that the tip of the papilla was reduced to a mass of cellular debris, which sloughed off into the pelvis (fig. 4). Paralleling the vascular changes, the interstitial tissue of the papilla always showed evidence of marked edema, and in many cases mucoid degeneration revealed metachromatically by toluidine blue. In several instances small epithelial protuberances were seen below the surface epithelium of the papilla. These apparently resulted from swelling due to edema of the underlying stroma. Telangiectatic veins filled with blood were present in these areas. Hermorrhage had occurred in the pelvis (fig. 5).

The collecting tubules showed some dilatation and occasional formation of cystlike spaces at the corticomedullary junction (fig. 6). There was moderate hyperplasia of the epithelium of the collecting tubules in the cases having mild lesions; the epithelial cells were tall and occasional mitotic figures were found. At the tip of the papilla glandlike formations with a suggestion of a papillary arrangement sometimes appeared (fig. 7). The pelvic mucosa was also hyperplastic, appearing as a thickened transitional epithelium, and sometimes it showed slight polypoid hyperplasia (fig. 4).

It is noteworthy that despite the severity of the lesions described there was little evidence of inflammatory reaction. Scattered granulocytes were occasionally found in the cortex, which also occasionally showed focalized lymphocytic infiltration. Some thickening of the basement

membrane of the glomerular tuft, with a slight amount of albuminous fluid in the glomerular capsule, was seen in only a few cases. It is surprising that in most cases the cortex of the kidney appeared to be normal. The total incidence of this lesion in its several stages was 82.5 percent. Definite necrotizing papillitis was found in 10 of the 40 animals of the experiment. In 23 of these animals several stages of the preceding vascular changes (congestion, thrombosis, edema) were found. The remaining 7 rats were free of kidney lesions.

Control animals, not a part of the experiment, kept on the basal diet in different colonies from time to time during the past 15 years in this laboratory with no mono-N-methylaniline hydrochloride, have shown neither gross kidney lesions nor bloody urine.

DISCUSSION

Certain microscopic lesions were found in rats fed mono-N-methylaniline hydrochloride, which was given in an attempt to produce hepatic tumors. No tumors were found in any of the rats, although they were given the substance for about 1½ years. However, an acute necrotizing papillitis of the kidney developed in some of the animals. The lesion consisted primarily of a vascular change that varied from congestion to thrombosis. There were concomitant interstitial edema, mucoid degeneration, and, terminally, necrosis of the tip of the papilla and hemorrhage. In addition, there were dilatation of some of the collecting tubules and moderate hyperplasia of their lining epithelium. In most animals, there was no associated inflammatory reaction in other parts of the kidney, and in the few instances in which an inflammatory reaction was seen, this should probably be considered as a coincident lesion occurring in older rats rather than a consequence of the lesion of the papilla.

Renal papillitis has been produced experimentally by several investigators using specific toxic substances: Levaditi in 1901 with vinylamin (*2*); Rehns in 1901 with tetra-hydroquinoline (*3*); and Oka in 1913 with bromethylamino-bromhydrate (*4*). More recently Burr and Burr (*5*), and later Borland and Jackson (*6*), have produced similar lesions in rats by the rigid exclusion of fat from the diet. A necrotizing papillitis in human beings has also been described. It is found most often in diabetic patients, and related to necrotizing pyelonephritis. In nondiabetic patients it is usually related to urinary obstruction and subsequent infection (*7–12*).

The pathogenesis of acute necrotizing papillitis is not yet well established either in experimental animals or in man. In the present experiment, the possibility of a direct and specific toxic action of the mono-N-methylaniline could be presumed to be the existing factor in the pathogenesis of this lesion. We must bear in mind, however, that the drug was given to the rats for a very long period of time and that the lesions found were of an acute nature, with remarkable vascular changes. Thrombosis and focal infarction were also found in the liver and in the spleen, whereas degenerative changes of the epithelium, often encountered in man, were infre-

quent or even absent in the rat. This strongly suggests the possibility of some type of sensitization to the drug with the final production of a vascular lesion comparable to the tissue necrosis of the Shwartzman phenomenon. There is little possibility that a dietary deficiency played any part in provoking the lesion because the diet given to the rats was adequate in all essential ingredients. The animals gained weight at a normal rate. The epithelial hyperplasia does not resemble any metaplastic or degenerative change that would indicate vitamin A deficiency.

We cannot offer an explanation for the calcium deposition of the thrombotic vessels and of the necrotic tissues. Calcium deposition was found in the epithelial cells at the corticomedullary junction of the kidney in animals fed a fat-free diet (*5*, *6*); in experimental hyperparathyroidism (*13*); and in magnesium deficiency (*14*). It would be interesting to know whether a state of hyperparathyroidism has been induced by the administration of mono-N-methylaniline. No tests were made to determine whether this condition existed in the animals of the present experiments.

SUMMARY AND CONCLUSIONS

An acute necrotizing renal papillitis is described in 10 of 40 rats fed mono-N-methylaniline hydrochloride for 1½ years in an attempt to induce hepatic tumors. Twenty-three of the experimental animals also showed what was interpreted to be early stages of the lesion. No tumors in the liver or elsewhere were found in these animals.

The condition above mentioned consisted primarily of a blood vascular change that varied from congestion to hyaline thrombosis of the smaller vessels with frequent deposition of a calciferous material in the thrombi. Necrosis of the tip of the renal papilla finally occurred. Similar vascular changes in the liver and spleen resulting in infarction or focal necrosis were found. Degenerative changes in the remainder of the kidney, often encountered in man with necrotizing papillitis, were infrequent or even absent in the rat.

It is tentatively suggested that the pathogenesis of this lesion involves sensitization to the drug with production of vascular lesions comparable to the tissue necrosis of Shwartzman's phenomenon.

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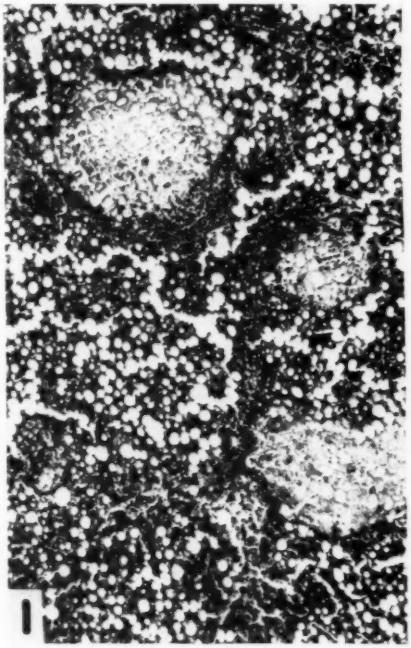
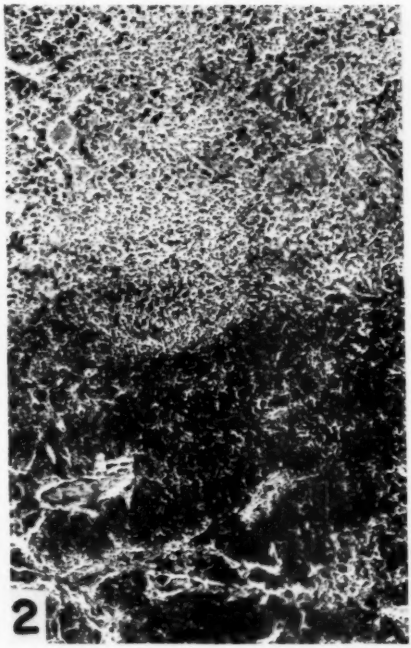
PLATE 74

FIGURE 1.—Liver of rat fed mono-N-methylaniline for 368 days. Marked fatty change. Several infarcts. Hematoxylin and eosin. × 100

FIGURE 2.—Spleen of rat fed mono-N-methylaniline for 668 days. Congestion and infarction of the spleen. Hematoxylin and eosin. × 100

FIGURE 3.—Kidney of rat fed mono-N-methylaniline for 666 days. Several small blood vessels with hyaline thrombi and calcium deposition. von Kossa silver impregnation for calcium. × 235





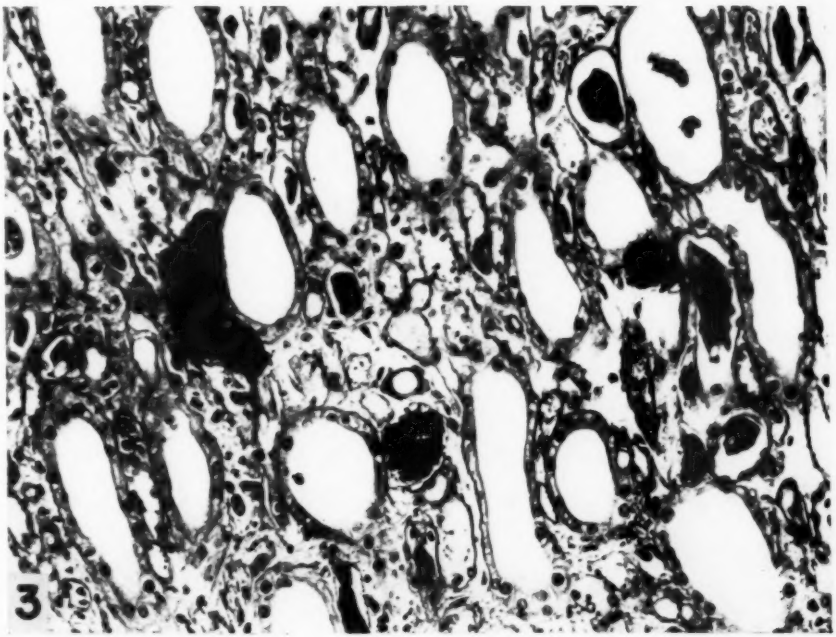


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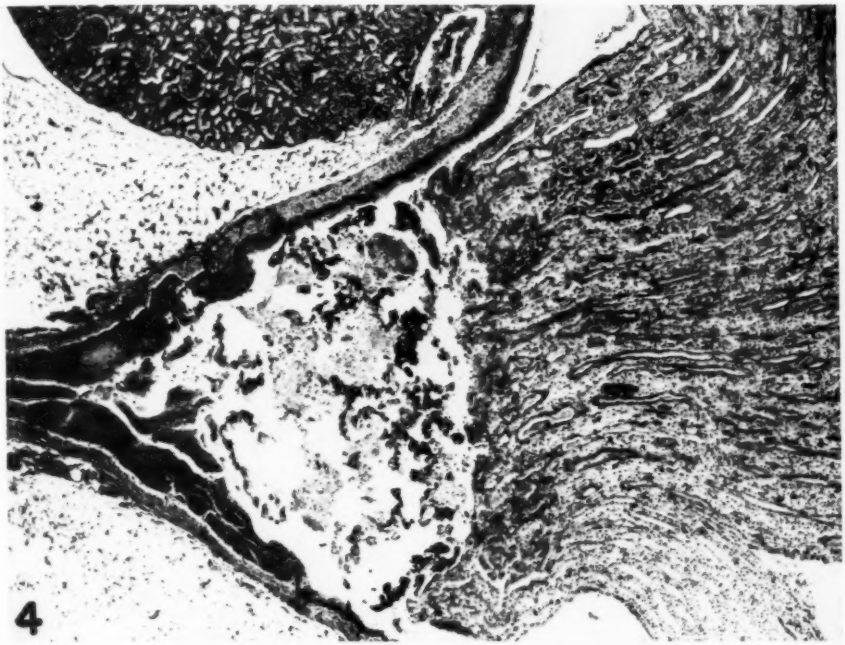
PLATE 75

FIGURE 4.—Kidney of rat fed mono-N-methylaniline for 661 days. Necrotic debris sloughed from the tip of the papilla. Slight hyperplasia of the pelvic mucosa. Hematoxylin and eosin. × 45

FIGURE 5.—Kidney of rat fed mono-N-methylaniline for 668 days. Extreme interstitial edema with protuberance of the surface epithelium of papilla. Hematoxylin and eosin. × 65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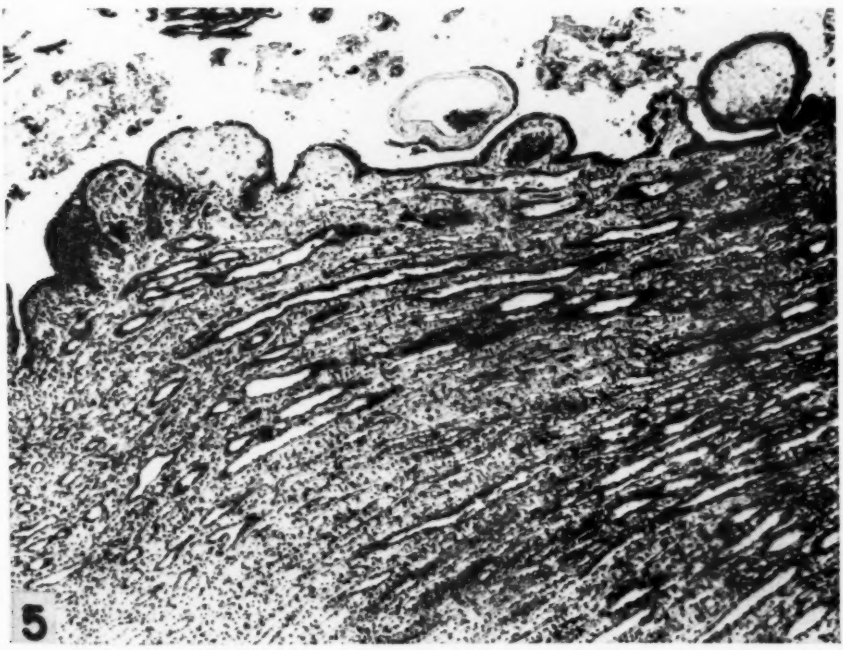
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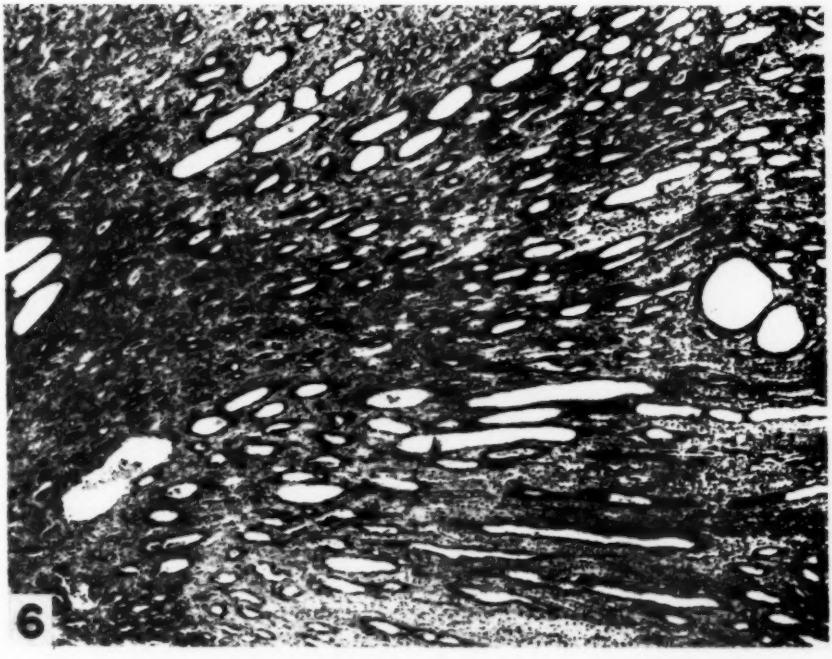
PLATE 76

FIGURE 6.—Kidney of rat fed mono-N-methylaniline for 668 days. Dilatation of collecting tubules. Hematoxylin and eosin. × 75

FIGURE 7.—Kidney of rat fed mono-N-methylaniline for 600 days. Moderate dilatation of collecting tubules. Hyperplasia of the epithelium, especially evident at the tip of the papilla. Hematoxylin and eosin. × 60



White and Mori-Chavez

INCREASE IN INCIDENCE OF LEUKEMIA IN HYBRID MICE BEARING THYMIC TRANSPLANTS FROM A HIGH LEUKEMIC STRAIN.[1] [2]

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The striking decrease in the incidence of leukemia after total thymectomy of ordinarily high leukemic strains of mice (*1–5*) indicates some relation of the thymus to neoplastic transformation of normal lymphoid cells. The precise mechanism through which thymic tissue influences the induction of leukemia is not understood. Furth and his associates (*1, 2*) interpret their findings to mean that the thymus contains "potentially malignant" cells that upon removal reduces the probability of the development of leukemia. Other investigators (*3–5, 6, 7*) believe that a more indirect role of the thymus exists in the genesis of certain leukemias. The present preliminary report concerns the influence of transplants of thymic tissue from a high leukemic strain on the incidence of leukemia in relatively low-leukemic test mice.

EXPERIMENTAL PROCEDURE

High leukemic RIL strain mice.—The incidence of leukemia in the RIL strain of mice (*3, 8, 9*) is approximately 85 percent, the mean age at death from leukemia being 7.6 months. The thymus appears to be initially and principally involved in the leukemic process in this mouse. Of 62 leukemic mice of this strain, 60 animals, or 96.8 percent, showed moderate to severe thymic involvement (*3*). In several cases the thymus was the only tissue found to be leukemic.

Low leukemic C3Hb mice.—The incidence of leukemia in strain C3H mice and in the C3Hb line of this strain is extremely low (*10, 11, 12*). The incidence of leukemia in the specific C3Hb line used in this study was approximately 1 percent.[4]

The F_1 generation of mice employed in this study was obtained by crossing females of the C3Hb line and males of strain RIL. These (C3Hb × RIL) mice represent second and third litters of females of the C3Hb line

[1] Received for publication October 4, 1951.

[2] With the technical assistance of J. H. Miller.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

[4] The strain C3H was obtained originally from Dr. W. E. Heston of this Institute. The C3Hb line has been maintained by brother × sister matings following foster nursing of a C3H strain litter of the F_{14} generation on a strain C57 black mother.

which were 6 weeks old when mated initially. Control animals were litter mates to those of the experimental group, the plan being to use one and two controls to three and four experimental mice of a single litter. The mice were kept in plastic cages, seven animals per cage, and given water and Derwood Mills' food cubes *ad libitum*.

The entire thymus of strain RIL mice, 4 to 5 weeks of age, was removed under aseptic conditions (*3*), and cut longitudinally into four sections, the largest sections measuring approximately 2 by 2 by 4 millimeters each. One fragment of the thymus was implanted subcutaneously in the right axillary region of (C3Hb×RIL) F_1 hybrid mice of the same age. A small mid-dorsal incision was made in the host animal, and a pocket prepared in the subcutaneous connective tissue of the axillary region. The fragment of tissue was then implanted in the pocket. Control mice were not operated upon. The experimental and control mice comprised nearly equal numbers of males and females.

RESULTS

Table 1 shows the information relating to incidence of leukemia and other pertinent data for the experimental and control animals. The incidence of leukemia in the F_1 mice bearing strain RIL thymic tissue is clearly significantly greater than that of the control mice. The difference of the incidences of leukemia in the 2 groups is 33.2 ± 13.9 percent; $P = 0.0166$. In the experimental mice 60.7 percent of the cases of leukemia had appeared before 10 months, after the implantation of the RIL thymus. The first case of leukemia in littermate controls, on the other hand, occurred at the tenth month. No sex difference in response was noted.

The leukemia of the treated mice was typically lymphocytic in type and infiltrated the superficial and internal lymph nodes, the spleen, the liver and in some cases the kidney and ovary. In the majority of cases the thymus of the host was involved. Typical, severe leukocytosis was a common characteristic, and leukemic cells were invariably found in the peripheral blood.

RECOVERY OF TRANSPLANTS OF THYMUS

Transplanted RIL thymic tissue was recovered from all but 2 of the 28 leukemic mice obtained thus far (figs. 1 and 2). In 1 animal extensive autolysis made recovery of the transplant virtually impossible; in a second test animal the thymic implant was obscured by a mammary tumor. The recovered fragments of thymic tissue ranged in size from 3 by 3 by 5 mm. to 10 by 10 by 15 mm., and the majority of transplants were readily palpable. Histologic study of the 26 recovered thymus transplants revealed that all but 2 were unquestionably leukemic.[5] Part of the recovered thymic tissue was used for histologic study, and the remainder of it inoculated subcutaneously into F_1 hybrid mice. In each of 17

[5] The author is grateful to Dr. Thelma B. Dunn, Laboratory of Pathology of this Institute, for her careful histologic diagnosis of these tissues.

such cases, the transplants grew profusely in the axillary region of the host, killing these animals in 15 to 39 days. In the terminal stages a generalized leukemia resulted in the host animals implanted with the recovered thymic tissue. Table 2 shows the extent of manifestations of leukemia in a representative sample of the 28 (C3Hb×RIL) F_1 hybrid mice bearing transplants of RIL thymic tissue and the results of transplantation of the recovered thymic and host leukemic tissue.

Transplants of recovered thymic tissue.—The transplantation characteristics of the recovered thymic fragments of 10 of these 17 cases of leukemic mice were then determined by transplantation of thymic tissue through 5 consecutive serial transfers. Recovered thymic tissue, in the first transfer, and transplanted thymic tissue, in transfers 2 through 5, was inoculated into the F_1 hybrid mice and into mice of the parental strains, RIL and C3Hb. It is clear from table 3 that the recovered thymic tissue of F_1 leukemic mice, although of RIL origin, behaves in transplant like (C3Hb×RIL) F_1 hybrid leukemic tissue, growing progressively in all of the host F_1 mice and generally failing to grow in either of the parental strains.[6] [See Bitner (*13*) and Little (*14*) for a summary of the results of transplantations of tumors arising in pure strain and hybrid mice.]

Transplants of host leukemic tissue.—In 19 instances an inoculum of infiltrated spleen and involved internal lymph node of leukemic F_1 mice, bearing transplants of RIL thymic tissue, was transplanted subcutaneously into normal F_1 mice. All 38 host animals employed developed leukemia and died in 10 to 54 days. Leukemic tissue grown subcutaneously was then used in 13 instances in serial transfer through 5 generations in the F_1 hybrid mice. Transplantation characteristics were determined at the various transfers, as discussed previously with recovered RIL thymic tissue, by inoculation into the (C3Hb×RIL) F_1 hybrid mice and mice of the parental strains, C3Hb and RIL. It is clear from table 3 that host leukemic tissues grow progressively only in the F_1 hybrid mice and fail to grow in either of the parental strains. This is to be expected of leukemic tissue that is of this specific (C3Hb×RIL) F_1 hybrid origin.

The fate of RIL thymic tissue transplanted to the right axilla of (C3Hb×RIL) F_1 hybrid mice was studied in a separate group from which the transplanted RIL thymic tissue was recovered at periodic intervals. The recovered tissue was fixed in Tellyesniczky's fluid, stained with hematoxylin and eosin, and sectioned semi-serially. A series of stages of degeneration of thymic round cells occur in such transplanted thymic tissue followed by regeneration of these cells. At 4 days after implantation nearly all of the round cells of the thymic transplant had disappeared. The remaining round cells showed extensive karyorrhexis and pyknosis. At 8 days only a reticular network of cells remained, and it appeared that this structure was hypertrophied, although this was by

[6] The exceptions listed in table 3 showing progressive growth in three animals of the RIL strain have been noted previously by Furth in transplantation studies of leukemic tissues obtained from F_1 hybrid mice of strains C3H and Ak. The strain RIL used in the present study is a derivative of strain Ak. [(Furth, J., Boon, M. C., and Kaliss, N.: On the genetic character of neoplastic cells as determined in transplantation experiments. Cancer Research 4 : 1–10, 1944.)]

TABLE 1.—*Incidence of leukemia in (C3Hb×RIL) F_1 hybrid mice bearing thymic transplants from the high leukemic RIL strain and in littermate controls*

Group	Leukemia		Leukemia by months										Mean age at death (months)	Number alive at 14 months [1]
	Number	Percent	6	7	8	9	10	11	12	13	14	15		
1) With RIL thymic tissue	28	60. 9	----	2	3	6	6	4	6	1	----	----	10. 0	16 (34.8 percent).
2) Controls	5	27. 7	----	----	----	----	1	1	----	2	1	----	12. 2	11 (61.1 percent).

[1] In group 1, 1 mammary tumor, an adenocarcinoma, appeared at 11 months and 1 animal died without a tumor at 13 months; in group 2, 1 mammary tumor, an adenocarcinoma, appeared at 11 months, and 1 animal died without a tumor at 11 months.

TABLE 2.—*Sample results of leukemic manifestations in (C3Hb×RIL) F_1 hybrid mice bearing transplants of RIL thymic fragments and results of transplantation of recovered thymic tissue and host leukemic tissue*

Mouse No.	Age at death from leukemia (months)	Transplanted RIL thymic tissue			Host organs infiltrated [1]				Growth of transplants [2]					
									Recovered thymus transplanted into—			Host leukemic tissue transplanted into—		
		Recovered	Size (millimeters)	Pathology	Lymph nodes	Liver	Spleen	Thymus	RIL	C3Hb	F_1	RIL	C3Hb	F_1
♀ 3	11	Yes	3 x 5 x 6	Leukemic	+	+	++	++	10−	10−	5+(31)	15−	10−	6+(36)
♀ 16	9	Yes	3 x 9 x 11	do	++	+	++	−	10−	10−	8+(38)	5−	5−	6+(21.4)
♂ 20	10	Yes	2 x 3 x 4	do	+++	+	++	+++	5−	5−	10+(20.2)	10−	10−	15+(28.4)
♀ 13	12	Yes	15 x 10 x 10	do	+	−	+	−	5−	5−	5+(25)	Not tested		
♂ 43	10	Yes	2 x 4 x 5	do	+++	++	++	+++	5−	5−	7+(37.6)	5−	5−	8+(22.0)
♀ 14	9	Yes	4 x 5 x 6	do	++	+	+++	+	5−	5−	11+(28.3)	10−	10−	10+(33.4)

[1] +++ Severe, ++ moderate, + slight and − no infiltration of tissues determined by microscopic observation.
[2] +=progressive growth of transplant resulting in death of the host animal. −=no growth of transplanted tissue. Numbers in parentheses indicate mean survival times in days of host animals.

TABLE 3.—*Fate in transplantation of 1) recovered transplants of RIL thymic tissue and 2) host leukemic tissues from (C3Hb×RIL) F_1 hybrid mice*

Group	Tissue used in transplant	Number transplanted into F_1 mice		Progressive growth (+); No growth (−)			
		Progressive growth	No growth	Number tested	Through 5 subsequent serial transfers in—		
					C3Hb/RIL	RIL	C3Hb
1	Recovered thymic transplants	[1] 17	0	10	107+; 0	[2] 2+; 60−	0+; 70−
2	Host leukemic tissue	19	0	13	126+; 0−	[3] 1+; 83−	0+; 82−

[1] In 16 cases the recovered thymic tissue and host leukemic tissue used for transplants came from the same animal.
[2] 1 of 5 strain RIL mice grew transplant progressively in the 3d transfer (tissue from hybrid mouse # ♀ 15) and 1 of 5 RIL mice grew transplant progressively in the 4th transfer (tissue from hybrid mouse # ♀ 80).
[3] 1 of 5 strain RIL mice grew transplant progressively in the 5th transfer (tissue from hybrid mouse # 56).

no means certain. There was no evidence of round cells at this time, and the original transplant had the appearance of an epithelial structure. At 10 and 14 days there was evidence of reappearance of numerous round cells, believed to be lymphocytes, and indication in some areas of arrangement in thymic lobules. The round cells were most prominent at first in the reticular network. By 18 days there was reconstitution of thymic structure (fig. 3 A and B) with beginning differentiation into cortex and medulla (fig. 4A) and the presence of bodies resembling Hassall's corpuscles (fig. 4B). Many newly formed blood vessels extended into the transplant. As determined by transplantation studies of the recovered thymic fragments, discussed above, the round cells in the reconstituted thymic tissue would appear to be lymphocytes derived from the F_1 hybrid host animal rather than cells derived from existing RIL (thymic) tissue.

DISCUSSION

The present investigation concerns an approach to the problem of the influence of the thymus in the development of spontaneous leukemia. Fragments of thymic tissue from the high-leukemic strain RIL were transplanted to F_1 hybrid host [produced by crossing low (C3Hb) and high (RIL) leukemic strains] mice. If cells of the RIL thymus are potentially malignant and represent transformation from normal to neoplastic, the leukemia that arises in the F_1 hybrid host should be, at least in certain cases, genetically RIL in origin. That is to say, the resultant leukemic tissues should grow progressively following transplantation in strain RIL mice as well as in (C3Hb $\times$ RIL) F_1 hybrid mice. If, on the other hand, transplanted thymic tissue is responsible for the induction of leukemia in the F_1 host, these leukemic tissues should be genetically host tissues, of C3Hb $\times$ RIL origin, and should grow progressively following transplantation only in F_1 hybrid mice of this specific genetic constitution. Since all host leukemic tissues tested, as well as all recovered thymic tissues, failed to grow in strain RIL mice but grew progressively in C3Hb $\times$ RIL mice, it must be assumed 1) that some of the cases of leukemia appearing in F_1 hybrid mice are induced leukemias, and 2) that RIL thymic tissue, in some manner, is responsible for the induction of this leukemia.

Among the 28 leukemic F_1 hybrid mice, 26 transplants of fragments of thymus were recovered. All except 2 were found to be undoubtedly leukemic as determined by histologic study, by transplantation, or by both methods. Four of the recovered transplants were massive growths, ranging in size from 3 by 9 by 11 mm. to 10 by 10 by 15 mm. These probably represent instances in which the transplant was involved initially in the leukemic process. In the remaining 20 cases, it was impossible to determine whether the transplants or host tissues were involved initially.

This method of transplantation of thymic tissue with the resultant development of leukemia in the transplants appears to offer an objective means of determining the origin of the lymphocyte-like cell of the thy-

mus. Two schools of thought have developed concerning the origin of these cells, each of which has upheld its contention through numerous reports during the last 40 years (*15–25*). The Maximow-Hammar group (*22, 23*) favors the interpretation that the round cells of the thymus are lymphocytic in nature, while the Maurer-Stöhr group (*24, 25*) claims an epithelial origin for these cells, believing also that they have the potentiality of reversion to their original epithelial form. The facts supporting these two theories have been obtained mostly by histogenetic studies of the degenerative and regenerative changes that follow auto-, homoio-, and hetero-transplantation of thymic tissue in various species of animal.

The evidence reported in the present paper favors the assumption that reconstituted thymic tissue, genetically RIL in origin but grown in (C3Hb×RIL) F_1 mice, is composed of reticular elements of RIL tissue and lymphocytes of F_1 hybrid tissue. It is assumed that following degeneration of the original thymic lymphocytes, infiltration of host lymphocytes occurred through the blood and lymphatic channels. Since 17 of the 26 recovered thymic transplants, which were leukemic, grew progressively with resultant leukemia only in the specific F_1 hybrids used in this study, and not in strain RIL mice, it would appear that this constitutes strong evidence that, under the conditions of these experiments, the lymphocytes of the reconstituted thymus are not epithelial in origin (*i. e.*, derived from persisting reticular elements) but rather that the lymphocytes of the host infiltrate the transplanted thymic tissue. Experiments are now in progress that attempt to define the mechanism of the influence of the thymus on the development of spontaneous leukemias.

SUMMARY

A statistically significant increase in the incidence of lymphocytic leukemia was found in (C3Hb×RIL) F_1 hybrid mice bearing transplanted fragments of thymic tissue from high-leukemic strain RIL mice.

Transplants of thymic tissue were recovered in 26 of the 28 leukemic F_1 mice and all except 2 were found to be leukemic.

These recovered thymic tissues grew progressively in F_1 hybrid mice only, and not in strain RIL mice (the strain of origin of the tissues). They behaved in transplant like the F_1 host leukemic tissues.

It is suggested that RIL thymic tissue, in some manner, induces leukemia in the F_1 hybrid host animal.

The conditions of these experiments provide an objective test for a consideration of the origin of the thymic lymphocyte. The available evidence favors the assumption that in reconstituted thymic tissue, lymphocytes have infiltrated from the F_1 hybrid host tissues and are not derived from existing reticular cells of the RIL thymic transplant.

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PLATES

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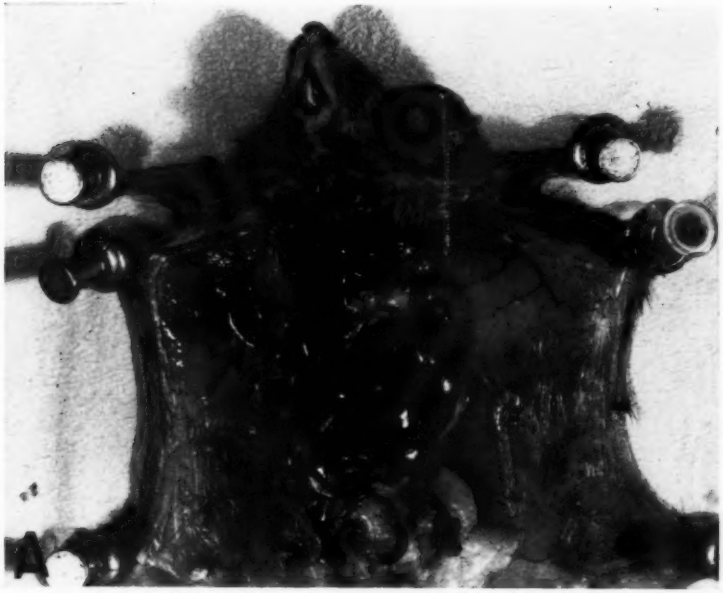
PLATE 77

FIGURE 1*A*.—Female (C3Hb × RIL) mouse No. 13, showing thymic transplant *in situ*. This mouse was leukemic at 12 months of age. See table 2 for details.

FIGURE 1*B*.—Thymic transplant (leukemic) from the same mouse. For comparison, longitudinal section of a normal thymus from a 4-week-old strain RIL mouse is shown.



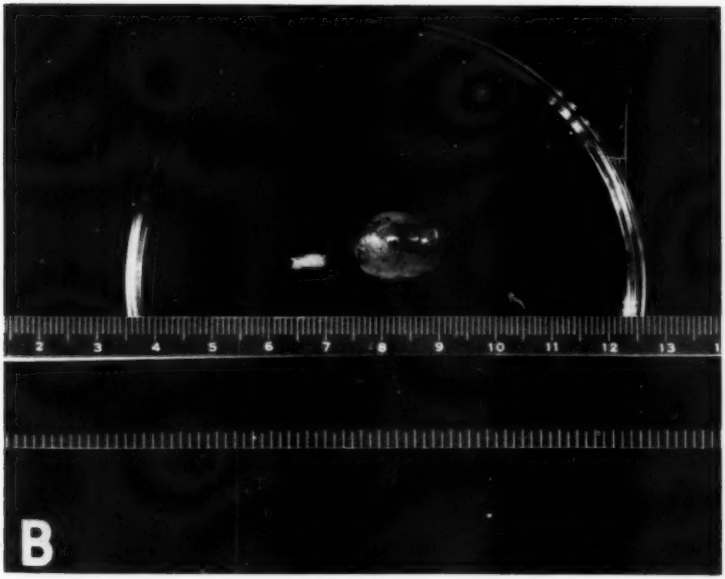


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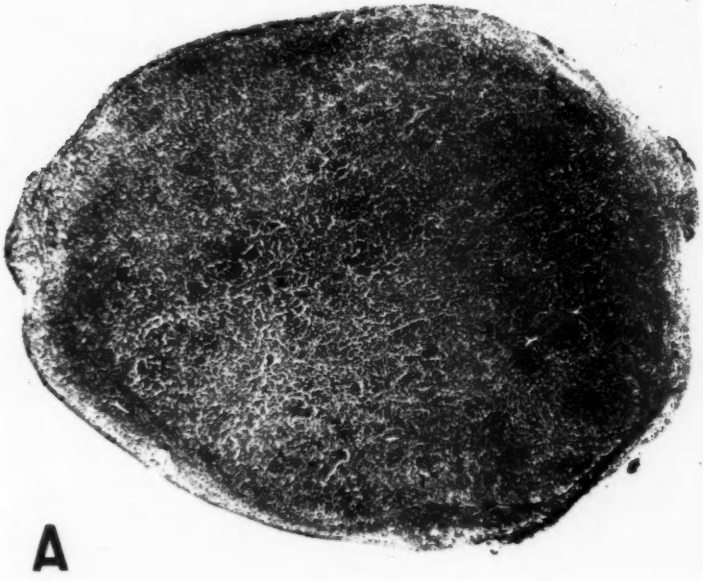
PLATE 78

FIGURE 2*A*.—Leukemic thymic transplant recovered from female F_1 hybrid No. 78, 10 months of age. This recovered transplant measured 4 by 6 by 6 mm. and grew progressively in the F_1 hybrid hosts. × 25

FIGURE 2*B*.—Same tissue. Note complete lack of typical architectural structure with profuse infiltration of leukemic cells. Note infiltration into capsule of thymic fragment. × 180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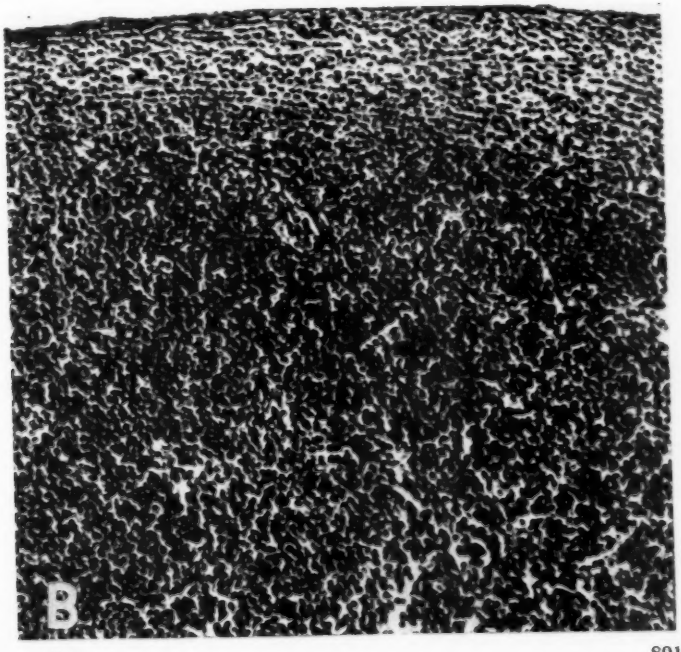
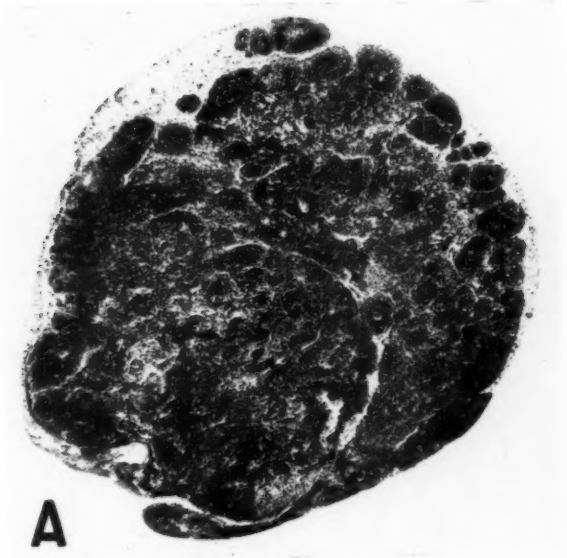

B

PLATE 79

FIGURE 3*A*.—Reconstituted RIL thymic transplant removed 18 days after transplantation into a (C3Hb × RIL) F_1 hybrid mouse. × 25

FIGURE 3*B*.—Same tissue. Note presence of many lobules and of lighter staining areas in most lobules apparently differentiating cortex and medulla. × 180



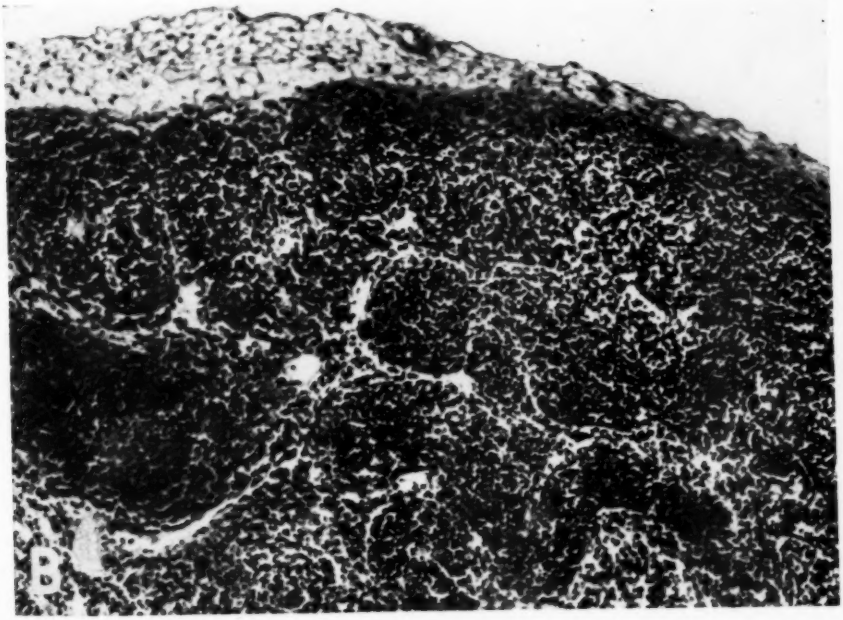


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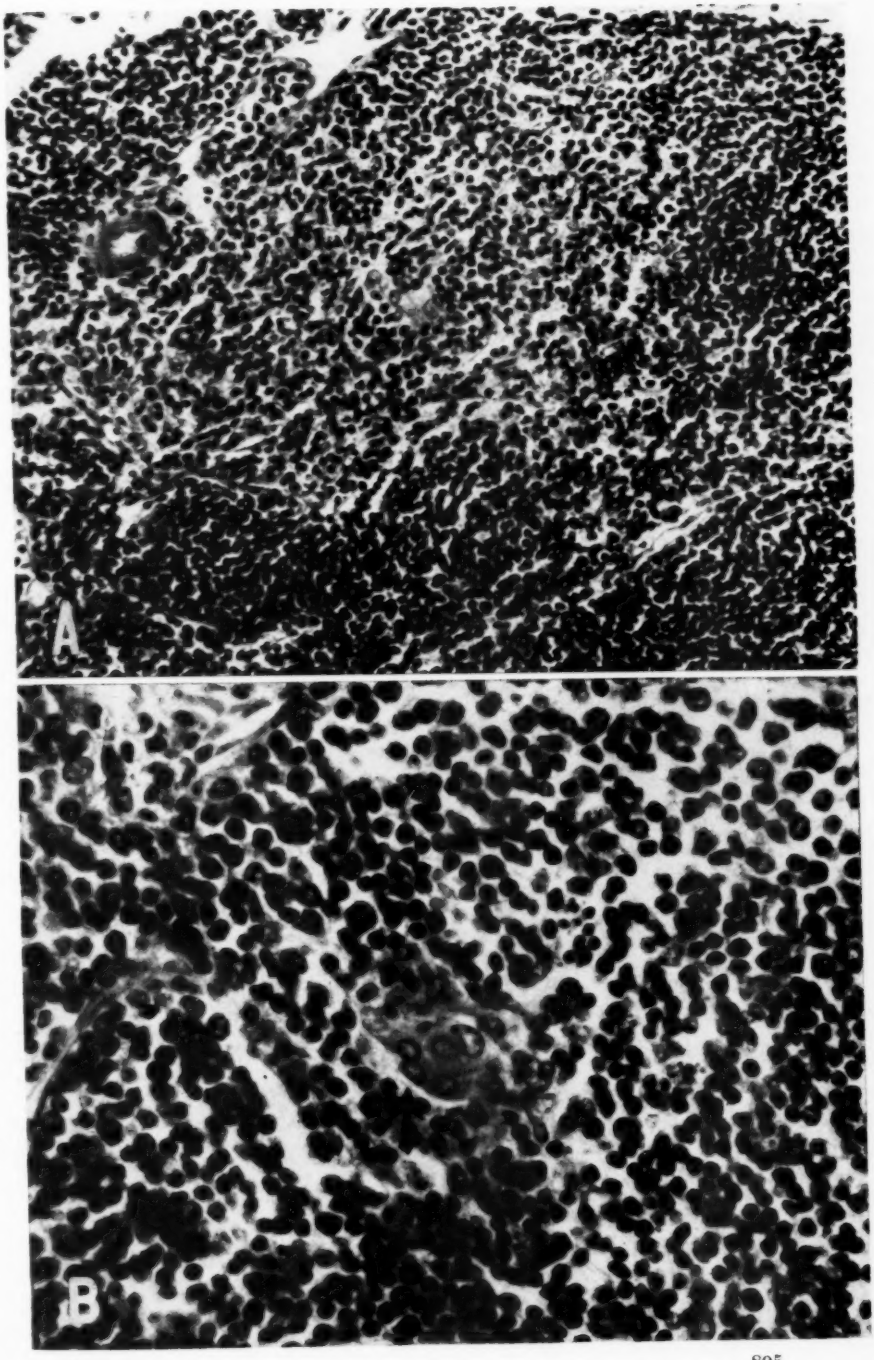
PLATE 80

FIGURE 4*A*.—Differentiation into cortical and medullary regions in a lobule of reconstituted thymic fragment shown in figure 3. Transplant removed 18 days after transplantation into an F_1 hybrid mouse. × 250

FIGURE 4*B*.—Structure resembling Hassall's corpuscle shown in same reconstituted thymic tissue at 18 days. × 610


A
B

THE EFFECT OF GUANAZOLO ON ANTIBODY FORMATION [1]

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INTRODUCTION

A depression of the reticuloendothelial system and a decrease in circulating antibody levels in tumor-bearing animals has been reported by several investigators (*1–3*). It has also been shown that antibody formation is inhibited by nitrogen mustard (*4*) and X-ray (*5*), both of which are known to be inciting as well as therapeutic agents for malignancy (*6–9*). Consequently, studies of other substances known to have one or more of these properties become interesting in an attempt to determine if they also inhibit antibody formation, to show whether or not tumor and antibody inhibition are related, and to determine, if possible, the manner in which these compounds act.

Since Kidder (*10*) first described the effect of guanazolo (8-azaguanine) [5-amino-7-hydroxy-1H-V-triazolo (d) pyrimidine] on animal tumors, numerous reports have appeared indicating that this antagonist of guanine inhibits the growth of certain tumors but does not affect the growth of other tumors in the same strain of mice (*14*). An effect of guanazolo on the lymphoid and myeloid tissues is suggested by the leukopenia induced by guanazolo (*11*). Further, guanine and guanylic acid have been employed for a number of years for the clinical treatment of agranulocytosis (*12*).

The following report on the effect of guanazolo on antibody formation in mice and rats is presented.

MATERIALS AND METHODS

Strains C (B alb C) and C3H mice of both sexes were used as test animals in the major part of these experiments. However, in studies of strain or species differences, some of the tests were done on mouse strains A, C57 black, DBA, and NH, and on male and female rats of strains M520 and Buffalo. The mice used were 2- to 6-months old and the rats 6- to 8-weeks old. Four or more animals were used in each group.

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Guanazolo [4] was dissolved in 0.01 N sodium hydroxide, guanylic acid and glutathione were dissolved in distilled water, and guanine hydrochloride was suspended in 5 percent gum arabic. All injections were made subcutaneously into the axilla, and if two substances were administered to the same mouse opposite axillae were used. A volume of 0.04 cc. per gram of body weight was used for all injections. Control animals received the same number and volume of injections of the vehicle. Unless otherwise specified the material tested was given once a day starting the day the antigen was injected and continued daily until the serum was harvested.

A 4 percent sheep red-cell suspension in 0.85 percent saline was used as the antigen in most of these studies. Five-tenths of a cubic centimeter of this suspension was injected intraperitoneally into mice, and 1.0 cc. was injected intraperitoneally into the rats. Five to 8 days later the animals were nembutalized and blood was obtained by cardiac puncture or by decapitation. The serum was removed and tested for hemolytic antibodies.

The hemolysin test consisted of 0.5 cc. of 0.85 percent saline, 0.5 cc. of sera in serial two-fold dilutions, 1.0 cc. of 1:20 guinea-pig complement and 0.5 cc. of a 1 percent sheep red-cell suspension. The tubes were incubated at 37° C. for 30 minutes and the turbidity was read as ++++ for no hemolysis and 0 for complete hemolysis. Intermediate turbidity was recorded as +, ++, or +++.

When the quantitative precipitin test was used, 2 mg. of bovine gamma globulin [5] per mouse was employed as the antigen. Two intraperitoneal injections of gamma globulin were given 3 days apart, and the serum was collected 8 days after the last injection.

Because of the small amount of antisera obtainable from mice the precipitin tests were performed with 0.5 cc. of serum to which a 0.1 cc. volume of bovine gamma globulin was added. After standing overnight in the refrigerator the precipitate was removed by centrifugation in the cold at 5,000×g. for 10 minutes. Subsequently, the addition of 0.1 cc. gamma globulin to the supernatant and centrifuging were repeated until no further precipitate resulted. Each 0.1 cc. of bovine gamma globulin contained 1 to 2 micrograms of nitrogen.[6]

After washing the combined precipitates three times in 0.85 percent saline it was taken up in distilled water and analyzed for total nitrogen by the Koch-McMeekin modification of Nessler's micro-Kjeldahl technique (*13*). The results were expressed in terms of antibody nitrogen per cc.

In a study of the effect of guanazolo on antibody formation in tumor-bearing animals, the C3H mammary tumor (C3HBA) and Lymphoma 1 in strain A mice reportedly inhibited by guanazolo (*11, 14*), were used. Also studied were Sarcoma 37 in strains C3H, C (B alb C) and C57 black

[4] 5-amino-7-hydroxy-1H-V-triazolo (d) pyrimidine (Guanazolo) was obtained from the Lederle Laboratory, Division of the American Cyanamid Company, Pearl River, N. Y.

[5] Fraction II, bovine plasma gamma globulin was obtained from the Chemical Research and Development Department, Armour & Co., Chicago, Ill.

[6] Although "univalent" antibody might not be detected by this method, the difference between the high- and low-antibody titers would be even greater by the standard quantitative percipitin test (*15*).

mice and Sarcoma 180 in strain C and C3H mice.[7] Neither of these tumors has been inhibited by guanazolo (*14*). The tumors were transplanted by trocar into appropriate mice, and when the tumors were about 1 cm. in diameter the animals were given 0.5 cc. of a 4 percent sheep red-cell suspension and divided into two groups. One group received 60 mg. of guanazolo/kg./day, while the control group received injections of 0.01 N sodium hydroxide.

To determine the importance of the adrenal glands in relation to the effects produced by guanazolo, a number of C and C3H female mice were adrenalectomized under nembutal anesthesia 3 to 5 days prior to treatment. These animals were divided into two groups, the treated group receiving guanazolo and the control group receiving 0.01 N sodium hydroxide. All the adrenalectomized animals were maintained by substituting 0.85 percent saline for drinking water. Because of the lethal effect of guanazolo (120 mg./kg./day) in adrenalectomized mice these experiments were performed using guanazolo (60 mg./kg./day).

The effect of guanazolo on passive antibodies was studied in mice which were injected intraperitoneally with 0.5 cc. antisera previously prepared in mice of the same strain. The mice were divided into two groups; one group received guanazolo 120 mg./kg./day starting 1 day before the antisera was injected while the other group remained untreated. Serum for the hemolysin test was harvested 48 to 72 hours later.

EXPERIMENTAL PROCEDURE

Numerous preliminary experiments on strain C mice indicated that 0.5 cc. of a 4 percent sheep red-cell suspension injected intraperitoneally was a satisfactory method for producing hemolysin. It was found that 5 to 8 days after injection of the antigen the antibody titer was at its highest level. Although reproducible results were obtained in each 2- to 6-month-old mouse tested, four mice were included in each group to minimize chance variation. No natural antisheep cell hemolysins in nonimmunized mice could be demonstrated. Because of the toxic effect of the compounds used and the resulting weight loss in treated animals, a series of tests was run on animals fasted for 96 to 120 hours. Although this fasting resulted in death for about half the animals, the survivors showed no decrease in their ability to produce antibodies. Further evidence that antibody inhibition was not due to generalized systemic toxicity is indicated by the lack of effect of such toxic substances as copper sulfate and sodium fluoride. When these compounds were given in sublethal doses under the same experimental conditions as guanazolo, no decrease in antibody production was observed.

The results in table 1 indicate that in strain C mice guanazolo at a dose level of 20 mg./kg. body weight/day, produced slight antibody inhibition and that larger doses of guanazolo proportionately reduced the

[7] Mammary tumor C3HBA and Sarcoma 37 were provided by Dr. M. K. Barrett, Dr. E. Lorenz supplied Lymphoma 1, and Dr. K. Sugiura gave us Sarcoma 180.

hemolysin titers. Marked inhibition was obtained in all strains with a dose of guanazolo of 120 mg./kg./day (table 2).

Several additional methods for treatment with guanazolo were tested. A single injection of guanazolo (120 mg./kg.) given simultaneously with the antigen did not depress antibody formation but gave a very slight indication of a stimulating effect.

Single injections of guanazolo were made in several groups of mice at varying time intervals after immunization. It was found that a single dose of guanazolo (120 mg./kg.) given on the 3d postimmunization day was most effective, and injections of guanazolo on the 4th or 5th post-immunization day were without effect. However, the single treatment with guanazolo on the 3d day after injection of the antigen depressed the antibody titer from 1:320 in the control animals to 1:160 in the treated animals.

Only slight, if any, reduction in hemolysin titers was observed in animals that received guanazolo (120 mg./kg./day) prior to, and no treatment after injection of the antigen.

In animals receiving guanazolo (120 mg./kg./day) 1 week before plus 1 week after injection of the antigen, and in animals receiving guanazolo (120 mg./kg./day) for 1 week after the injection of the antigen the degree of depression of the hemolysin titer was shown to be equal. Both methods resulted in greater reduction of the hemolysin titer than did the other schedules tested.

TABLE 1.—*Effect of guanazolo on hemolysin production in strain C mice*

Dose of guanazolo in mg./kg./day	Antisera dilutions							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
NaOH 0.01 N (control)	0	0	0	0	0	0	+++	++++
10	0	0	0	0	0	0	+++	++++
20	0	0	0	0	0	++	+++	++++
40	0	0	0	0	+++	++++	++++	++++
80	0	0	0	+++	++++	++++	++++	++++
120	++	+++	++++	++++	++++	++++	++++	++++

Guanine hydrochloride alone did not affect the antibody titer but when given simultaneously with guanazolo the two compounds tended to depress the hemolysin titer more than did guanazolo alone. Even in the ratio of 5 parts by weight of guanine hydrochloride to 1 guanazolo (guanine hydrochloride, 600 mg./kg./day, to guanazolo, 120 mg./kg./day) it was impossible to reverse the hemolysin-depressing effect of guanazolo with guanine (table 3).

Guanylic acid in a ratio of 2½ guanylic acid to 1 guanazolo (guanylic acid, 300 mg./kg./day, to guanazolo, 120 mg./kg./day) showed a slight tendency toward reversing the antibody depressing effect of guanazolo

TABLE 2.—*Effect of guanazolo at a dose level of 120 mg./kg./day on the hemolysin titer in various strains of mice*

Strain of mice	Group	Antisera dilutions							
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
DBA	Guanazolo	++++	++++	++++	++++	++++	++++	++++	++++
	Control	0	0	0	0	+++	++++	++++	++++
C3H	Guanazolo	++++	++++	++++	++++	++++	++++	++++	++++
	Control	0	0	0	0	0	+++	++++	++++
C	Guanazolo	++	+++	++++	++++	++++	++++	++++	++++
	Control	0	0	0	0	0	0	+++	++++
C57 black	Guanazolo	++++	++++	++++	++++	++++	++++	++++	++++
	Control	0	0	0	+++	++++	++++	++++	++++
A	Guanazolo	++++	++++	++++	++++	++++	++++	++++	++++
	Control	0	0	0	0	0	++	++++	++++
NH	Guanazolo	++++	++++	++++	++++	++++	++++	++++	++++
	Control	0	0	0	0	+++	++++	++++	++++

(table 3). Increasing the amount of guanylic acid did not enhance its effect.

It has been reported (*15*) that glutathione protects animals against the damaging effects of X-ray presumably by protecting the S–H containing enzyme systems. For this reason glutathione was tested for its ability to protect the antibody-forming system from the effects of guanazolo. It was found that glutathione in a ratio of 2⅔ to 1 guanazolo (glutathione, 320 mg./kg./day, to guanazolo, 120 mg./kg./day) reversed the inhibiting effect of guanazolo to approximately the same degree as it was reversed by guanylic acid (table 3).

In C3Hb mice bearing transplanted mammary tumor C3HBA, it was found that guanazolo, 60 mg./kg./day, almost completely inhibited antibody formation (table 4) in contrast to the 100–120 mg./kg./day necessary to produce the same degree of inhibition in nontumor-bearing animals (table 2). As has been previously noted (*2*), there was some depression of antibody titer in the tumor-bearing animals before treatment with guanazolo. In a similar test using C57 black mice bearing Sarcoma 37 it was found that the antibody titer was so low in the control mice bearing tumors that little effect of the guanazolo could be demonstrated (table 4). The same depressing effect of guanazolo on the antibody titer was noted with strains C and C3H mice bearing Sarcoma 180, and strain A mice bearing Lymphoma 1.

For purposes of comparison guanazolo was also administered to rats, since rats have been reported to differ from mice in their metabolism of guanine (*16*). Guanazolo had no effect on the hemolysin titer of rats when administered at the rates of 60 mg./kg./day or 150 mg./kg./day.

Guanazolo had no effect on the antibody titer of passively immunized mice (table 5) nor did it show an *in vitro* effect when 3 mg. of guanazolo were added to aliquots of antisera before performing the hemolysin test. Guanylic acid, guanine hydrochloride, and glutathione were also without *in vitro* effect when added in 3-mg. amounts.

TABLE 3.—*Effect of guanine hydrochloride, guanylic acid, and glutathione on hemolysin titers showing the antibody inhibition effect of guanazolo (120 mg./kg./day) in C3H mice*

Daily treatment	Antisera dilutions						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Guanazolo, 120 mg./kg	++++	++++	++++	++++	++++	++++	++++
Gum arabic, 5 percent	0	0	0	0	0	+++	++++
NaOH 0.01 N	0	0	0	0	0	+++	++++
Guanine hydrochloride, 600 mg./kg	0	0	0	0	0	+++	++++
Guanylic acid, 625 mg./kg	0	0	0	0	0	++	++++
Glutathione, 640 mg./kg	0	0	0	0	0	++++	++++
Guanazolo, 120 mg./kg. + Guanine hydrochloride, 600 mg./kg	++++	++++	++++	++++	++++	++++	++++
Guanazolo, 120 mg./kg. + Guanylic acid, 300 mg./kg	0	++	++++	++++	++++	++++	++++
Guanazolo, 120 mg./kg. + Glutathione, 320 mg./kg	0	++	++++	++++	++++	++++	++++

TABLE 4.—*Effect of guanazolo (60 mg./kg./day) on hemolysin titer of mice with tumors*

Strain of mice	Group	Tumor	Antisera dilutions								
			1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
C3H	Tumor+guanazolo	C3HBA	++	++++	++++	++++	++++	++++	++++	++++	+++++
	Tumor		0	0	0	0	++	+++	++++	++++	+++++
	Guanazolo	No tumor	0	0	0	+	+++	++++	++++	++++	+++++
	Control	do	0	0	0	0	0	0	±	+++	+++++
C3H	Tumor+guanazolo	Sarcoma 180	0	0	++	++++	++++	++++	++++	++++	+++++
	Tumor		0	0	0	0	0	+++	++++	++++	+++++
	Guanazolo	No tumor	0	0	0	0	+++	++++	++++	++++	+++++
	Control	do	0	0	0	0	0	0	0	+++	+++++
C	Tumor+guanazolo	Sarcoma 180	0	0	0	0	+++	++++	++++	++++	+++++
	Tumor		0	0	0	0	0	0	+	+++	+++++
	Guanazolo	No tumor	0	0	0	0	+++	+++	++++	++++	+++++
	Control	do	0	0	0	0	0	0	0	++	+++++
A	Tumor+guanazolo	Lymphoma No. 1	++++	++++	++++	++++	++++	++++	++++	++++	+++++
	Tumor		0	0	0	0	±	+++	++++	++++	+++++
	Guanazolo	No tumor	0	++	++++	++++	+++	++++	++++	++++	+++++
	Control	do	0	0	0	0	0	0	±	+++	+++++
C57 black	Tumor+guanazolo	Sarcoma 37	++++	++++	++++	++++	++++	++++	++++	++++	+++++
	Tumor		0	++	++++	++++	++++	++++	++++	++++	+++++
	Guanazolo	No tumor	0	++++	++++	++++	++++	++++	++++	++++	+++++
	Control	do	0	0	0	+++	++++	++++	++++	++++	+++++

TABLE 5.—*Effect of guanazolo (120 mg./kg./day) on hemolysin titer of sera from strain C mice passively immunized*

Antisera from—	Antisera dilutions			
	1:10	1:20	1:40	1:80
Guanazolo-treated mice	0	0	++	++++
Untreated control mice	0	0	++	++++

TABLE 6.—*Effect of guanazolo (120 mg./kg./day) on the precipitin titer in strain C mice*

Mice treated with—	μg. N/CC [1]
Guanazolo	14. 4
NaOH 0.01 N	38. 4
Untreated control	36. 4

[1] These values represent the nitrogen value after subtracting the nitrogen added as gamma globulin.

Adrenalectomy *per se* did not affect the antibody titer and, although adrenalectomized mice tolerated guanazolo less well than nonadrenalectomized mice, the same degree of depression of the antibody titer existed in both groups.

Precipitin titers, like hemolysin titers, were reduced by guanazolo (table 6).

DISCUSSION

The manner in which guanazolo depresses tumor growth and antibody formation and the relationship, if any, between these two phenomena are not readily apparent. Among the explanations that might be suggested to clarify this dual role of guanazolo are 1) toxicity to the animal, 2) stimulation of organs of internal secretion, or 3) an interference with one or more mechanisms involved in tumor growth and antibody production which may or may not have a direct relation to each other.

It has previously been shown that toxicity with its resulting lowered food intake and weight loss will cause some tumors to regress (*17*). Therefore, since Goldin *et al.* (*11*) have shown guanazolo to possess toxic properties it is conceivable that its inhibiting effect on both tumors and antibodies may be a result of its toxic properties. It would appear from the results, however, that antibody formation is not decreased by severe fasting or treatment with other chemicals in just sublethal doses. These results are in agreement with the findings of Goldin *et al.* and indicate that the lower antibody titers and tumor inhibition induced by guanazolo are not a result of generalized systemic toxicity.

The possibility also exists that guanazolo depresses antibodies by stimulation or suppression of one or more secretions of the endocrine system. Because of studies previously reported (*18*) linking the adrenal glands to antibody formation, and especially because of Law's work (*19*) showing adrenal hyperplasia in guanazolo-treated animals and Germuth's work (*20*) indicating lowering of antibody titers by Compound E, the relation of the adrenal glands to guanazolo and antibody formation was investigated.

It was found that adrenalectomized mice had a lowered tolerance for guanazolo. This was, perhaps, to be expected inasmuch as adrenalectomy is known to decrease the tolerance for toxic substances (*22*). Except for this lowered tolerance, however, adrenalectomized mice responded as well as nonadrenalectomized mice when treated with guanazolo, and the antibody titers of the two groups were depressed equally. These findings indicate that the action of guanazolo, therefore, is not by way of stimulation of the adrenal gland; but this does not rule out a possible alteration of innumerable other substances, known and unknown (hormones, breakdown products of tissue destruction, *etc.*) which may be involved in both tumor growth and antibody formation.

It seems evident from these studies that the depressing effect of guanazolo occurs at some stage in the process of antibody formation, since antibodies *in vitro* and passive antibodies *in vivo* were not affected by guanazolo.

The chemical nature of guanazolo suggests that its action may be due to interference with the metabolism of guanine or guanylic acid to which it may be antagonistic. This assumption is supported in part by the fact that rats, whose guanine metabolism differs from that of mice (*16*), show no inhibition of antibody formation when treated with guanazolo. A further indication that this may be correct is the slight reversal effect of guanylic acid on the lowered antibody titer of guanazolo-treated mice. It also seems possible on this basis to explain both tumor and antibody inhibition, since both processes might be adversely affected by an alteration of guanylic acid metabolism. Such an interference with guanylic acid metabolism might well be reflected in nucleic acid synthesis in the mouse. The work of Skipper *et al.* (*21*), who reported that guanazolo interferes with nucleic acid synthesis, suggests that the tumor and antibody-inhibiting effect of guanazolo might be explained on this basis.

An inconsistency in this explanation arises, however, when one considers that not all tumors are inhibited by guanazolo. It is highly improbable that all tumors, which are *a priori* synthesizing nucleic acids, should not be affected if interference with nucleic acid synthesis is the correct explanation for the action of guanazolo. Kidder (*14*) has explained this paradox by suggesting that only those tumors that require an exogenous source of guanine are affected by guanazolo. At the moment this seems the most likely explanation.

Interference by guanazolo with an S-H enzyme system may be implied by the reversal of the antibody-inhibiting effect of guanazolo noted for glutathione. It has been shown that the lethal effect of X-ray (*15*) can be reduced by treatment with glutathione, presumably because glutathione protects the S-H enzyme systems. Therefore, it is possible to infer that guanazolo, like X-ray, not only inhibits antibody formation and some tumors, but also attacks an S-H enzyme system.

It seems possible in view of this varying susceptibility of tumors to guanazolo that a strain difference might exist in mice that would help explain the differences noted. Therefore, an attempt was made to cor-

relate the response of the antibody-forming system in several strains with the response of their tumors. Results indicate, however, that there is no apparent relationship between tumor inhibition and antibody inhibition, inasmuch as antibodies were equally decreased in all strains of mice whether or not the tumor carried in that strain was affected. This finding, further borne out by reports that one tumor in a strain of mice is inhibited while another tumor in the same strain is not inhibited (*14*), strongly suggests that the tumor-inhibiting properties of guanazolo are related to the tumor and not to the host, and that tumor inhibition and antibody inhibition in mice are related only indirectly. The possibility exists, however, that the effect of guanazolo is upon the host, and the tumors vary in their susceptibility to this change in the host.

It is interesting to observe the striking correlation between the dose response curve (table 1) for the antibody-depressing effect of guanazolo and the curve presented by Kidder *et al.* (*14*) for tumor inhibition.

There appeared to be a summation of the inhibiting effects when guanazolo was administered to tumor-bearing animals. Evaluation of the further depression of the antibody-forming system when guanazolo is administered to tumor-bearing mice is difficult to make because of the already decreased antibody titer in control animals bearing tumors. This observation can perhaps best be expressed by saying that a dose of guanazolo (60 mg./kg./day) which gives approximately 50 percent antibody depression in normal animals will, when administered to tumor-bearing animals, whose antibody titer is about 25 percent depressed by the tumor, result in about 75 percent antibody depression.

In general, it would appear that guanazolo does effectively reduce antibody titers in the mouse but not in the rat, and evidence available at this time suggests that this depression is achieved by interference with guanylic acid metabolism. This is the same mechanism that has been suggested to explain the inhibitory effect of guanazolo on some tumors.

SUMMARY

Guanazolo (5-amino-7-hydroxy-1H-V-triazolo (d) pyrimidine) reduces hemolysin and precipitin antibody titers in several strains of inbred mice. The effective dose range is comparable to the dosage reported to produce tumor inhibition. No such antibody inhibition was detected in rats. This depression of antibody titers in mice is attributed to interference with antibody formation, since guanazolo did not depress antibodies *in vitro* nor did it depress passive antibodies. The depressing effect was not reversed by guanine but was reversed to a slight degree by guanylic acid and glutathione. Adrenelectomy did not reverse the reduced antibody titer although guanazolo seemed to be more toxic for the adrenalectomized mice than for nonadrenalectomized mice. These findings are discussed in relation to the possible mechanism of action of guanazolo.

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STUDIES ON THE TRANSMISSION OF THE MOUSE MAMMARY TUMOR AGENT BY THE MALE PARENT [1]

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It is generally agreed that the mammary tumor incidence in female hybrid mice procured by mating low-cancer-strain females to high-cancer-strain males is higher than in females of the maternal strain, but definitely lower than in females of the paternal strain (*1*). In a series of experiments Andervont (*2,7*) and Andervont and Dunn (*3–6*) have studied the question to what extent the mammary tumor agent is involved in the development of the mammary tumors, and if so, how it gained access to the hybrids. In their publications Andervont and Dunn have also discussed the general aspects of this problem. There is as yet no satisfactory explanation for the development of the mammary tumors in the F_1 hybrids between low-cancer-strain females and high-cancer-strain males. One possibility is that these hybrids get the mammary tumor agent from the father. It is known that the agent is present in the blood and in practically all organs that have been tested of both females and males of high-cancer strains (*8,9*). In what manner would a transmission of the agent be possible? Susceptible animals in bodily contact with high-cancer-strain mice did not develop mammary cancer (*8*). The agent could not be found in the excreta, urine, or feces of high-cancer-strain mice (*10*). Contamination with the agent through the excreta is, therefore, improbable. These negative results call attention to the sperm as a possible means of transmission of the agent at copulation. It was indeed possible to demonstrate the agent in the sperm from the cauda epididymidis of high-cancer-strain males (*11*), and also in the seminal vesicles (*3*). In their publications, Andervont and Dunn have given some data that make the concept of a transmission of the agent by the male parent probable. In cross-breeding experiments with the low-cancer strain C57 black and the high-cancer strain RIII, Foulds (*12*) has found likewise a series of cases that were interpreted as a transmission of the agent by the father. Recently Bittner (*13*), in experiments with the C strain, has also demonstrated that a transfer of the agent by the male parent is possible. In this paper the results have been reported of experiments to investigate

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the possibility of a transmission of the mammary tumor agent by the male parent in hybrids with the dilute-brown strain.

MATERIALS AND METHODS

All mice were raised in this Institute, fed commercial pellets and grain (wheat), a small handful twice a week, and tap water *ad libitum*. The animals were kept four or five in a box. Observations were made at weekly intervals, tumors being noted as appearing on the date when first palpable. Histologic diagnoses were made in all cases. The tissues were fixed in Susa solution and stained with hematoxylin and azophloxin. The following strains and hybrids were used:

d=dilute-brown strain (Little).

dz=dilute-brown strain without mammary tumor agent, procured by taking the young from the uterus of a pregnant d mouse and foster nursing them on a female of the B strain. The young were further bred by constant brother-sister mating.

B=C57 black strain (Little).

020=020 Leeuwenhoek-Huis strain.

Bd=BdF_1-hybrids from mating B females to d males.

Bdz=BdzF_1-hybrids from mating B females to dz males without the agent.

Od=OdF_1-hybrids from mating 020 females to d males.

Odz=OdzF_1-hybrids from mating 020 Leeuwenhoek-Huis strain females to dz males without the agent.

Bd×d=(Bd×d)BC-hybrids from mating Bd females to d males.

Bd×dz=(Bd×dz)BC-hybrids from mating Bd females to dz males.

Odz×dz=(Odz×dz)BC-hybrids from mating Odz females to dz males.

I. The Mammary Tumor Agent in the Sperm [2]

Experiment A.—Injection of the sperm from the cauda epididymidis of the high-cancer strain dilute brown into infantile susceptible hybrids gives rise to a high percentage of mammary tumors in these hybrids (*11*). The sperm, therefore, contains the mammary tumor agent. Naturally it has to be ascertained whether or not after injection into susceptible hybrids the mammary tumor agent from the sperm is transmitted to the young with the milk. Infantile Odz females were injected intraperitoneally with the contents of the cauda epididymidis one to three times as previously described (*11*). At the age of 6 months these hybrids were mated with dz males. The daughters (♀ Odz × dz) were force-bred with their brothers for 1 year and then observed for the appearance of mammary tumors. Controls were Odz hybrids untreated and treated in the same manner after injection with sperm from males of the noncancer strains C57 black and 020. The results are summarized in table 1 *A* and 1 *D*.

[2] Throughout this paper the term "sperm" denotes the spermatozoa containing secretions of the testis and the epididymidis. "Spermatozoon" denotes the male germ cell.

TABLE 1.—*Mammary tumor incidence in susceptible, with sperm injected, hybrids and their untreated daughters*

♀ Odz	Mothers injected				Daughters from breeding with ♂dz			
	Total number	With mammary tumors		Nontumor-bearing—average age at death in days	Total number	With mammary tumors		Nontumor-bearing—average age at death in days
		Number	Average age in days with tumor			Number	Average age in days with tumor	
A. Infantile hybrids injected with sperm from ♂d:								
3x	13	11	454	656	71	60	360	540
1–2x	4	0	------	739	27	4	473	634
3x with sperm from ♂B and ♂020	10	0	------	------	84	9	487	598
B. Adult hybrids 3× injected with sperm from ♂d	16	11	462	684	137	104	292	476
C. Adult hybrids 12× injected with sperm of high-cancer males	11	7	595	556	145	95	323	484
D. Controls untreated	12	1	636	629	105	10	433	525

The daughters of the hybrids injected with sperm from males with the agent (♂d) have a high percentage of mammary cancer, whereas in the control group after injection of sperm from males without the agent (♂B and ♂020) only a few mammary cancers were found. Four infantile susceptible hybrids were injected only one or two times with the sperm of the high-cancer strain males; none developed a mammary tumor, and there was also no transmission of the agent to the daughters.

Although this group is small, it is probable that the concentration of the agent with one or two sperm injections was too small to give rise to mammary tumors in the injected animals and their daughters.

Experiment B.—This experiment was done to investigate whether mammary tumors also arise if the injection of the sperm takes place in adult hybrids. Odz hybrids 3 months of age were injected three times with sperm of d males in the same manner as the infantile animals. After the injection of the sperm the hybrids were bred with dz males. The daughters were force-bred with their brothers until they were 12 months of age and then put aside and watched for the appearance of mammary tumors. The experiment was terminated when the youngest daughter was 18 months old. The results are summarized in table 1 *B*. Injection of the sperm of high-cancer males three times into adult susceptible hybrids gives rise to mammary tumors in these hybrids. The agent is transmitted to their daughters, which have a high incidence of mammary tumors.

Experiment C.—This experiment was done in the same manner as in experiment B, only the injection of the sperm was repeated 12 times, twice weekly for 6 weeks. Besides the sperm of d males also the sperm of other high-cancer strain males was injected. The other high-cancer

strain males were from the RIII (Paris)—the White-Label—and the A Strong strain. The tumor incidence in these strains in our laboratory in the last few years has been reported in a recent paper (*14*). The results of this experiment are summarized in table 1 *C*. As in experiment B, the hybrids injected with sperm had a high incidence of mammary tumors, and so had their untreated daughters.

Experiment D.—This is the control experiment to those of A–C. Untreated Odz females were bred with dz males, and the daughters preserved in the same manner as in experiments A–C. Only a small percentage of mammary cancer in the daughters was observed.

Conclusion: *Experiments A–D.*—Development of mammary cancer was found not only in the case of injection of the sperm from high-cancer strain males into infantile susceptible hybrids (♀ Odz), but also after identical treatment of adult hybrids. All hybrids transmitted the agent to their daughters. Consequently the agent in the sperm behaves like the agent in the mother's milk.

II. Comparison of the Mammary Tumor Incidence in Low-Cancer Strain Females After Force-Breeding With Males With and Without the Agent

This experiment was done with females of the 020 Leeuwenhoek-Huis strain, a low-cancer strain that is susceptible to the mammary tumor agent and with females of the dz line.

At weaning age litter mates were divided into two groups. The first group of the 020 females were force-bred with males of the same 020 strain, the second group with d males. The first group of the dz line were force-bred with males of the dz line without the agent; the second group with males of the d strain with the agent. Four females from each group were force-bred with one male. Immediately after birth the young were removed. The force-breeding was continued until the end of the breeding period. The females were then kept four in a cage and watched for the appearance of mammary tumors. The tumor incidence is given in table 2.

Table 2.—*Mammary-tumor-incidence after force-breeding with males with and without the agent*

	Total number	Average number of litters	With mammary cancer			Without mammary tumor	
			Number	Percent	Average tumor-age in days	Number	Average age at death in days
♀ 020 force-bred with ♂ 020 (without agent)	109	8	13	12	581	96	605
with ♂ d (with agent)	59	10	5	8	565	54	554
♀ dz, litter mates force-bred:							
With ♂ dz (without agent	105	6	24	23	531	81	575
With ♂ d (with agent)	92	6	16	17	552	76	569

Conclusion.—In each group of these two strains a small incidence of mammary tumors was found. The percentage of mammary tumors is

not higher in the group force-bred with males with the agent. The results therefore give no proof that in these strains a transmission of the agent with the sperm at copulation took place.

III. Mammary Tumor Incidence in Susceptible Hybrids Force-Bred With Males With and Without the Agent and in Their Foster Daughters

Two experiments were done. The first with Od females [F_1 (♀020 + ♂d)]. Litter mates were divided into 2 groups. The first group was force bred with d males with the agent; the second with males of the 020 strain without the agent. After 10 pregnancies, after which the young were discarded, foster daughters were added to the young, 2 to each litter. Odz females, which were highly susceptible to the agent, served as foster daughters in both groups. After weaning age these foster daughters were force-bred with untreated Odz males till the age of 1 year and then watched for the appearance of mammary tumors.

In the second experiment Bd females were used in the same manner as the Od females. Litter mates were divided into 4 groups. The first group was force-bred with d males with the agent, the other 3 groups were force-bred with males without the agent: B males, 020 males, and Bd males. After 10 pregnancies the females were allowed to nurse their young. To each litter were added 2 foster daughters, which were preserved in the same manner as in the first experiment. The mammary tumor incidence is given in table 3.

Conclusion.—As in experiment II the hybrid mothers force-bred by males with the agent have no higher incidence than their sisters, which were force-bred with males without the agent. There is also no difference in the mammary tumor incidence of the foster daughters. An accumulation of tumors in certain groups of foster daughters could not be observed. Therefore a transfer of the agent by the male parent could not be demonstrated in the mothers nor in the foster daughters.

IV. Mammary Tumor Incidence in Sterilized Hybrids Mated With Males With and Without the Agent

If transmission of the agent at copulation is possible the chance of "infection" of the mother must be greater with a greater number of copulations. Under normal conditions a maximum of one copulation in 3 to 4 weeks is possible. If fertilization is prevented a copulation may be expected at the end of the pseudopregnancy, *i. e.*, after 12 to 14 days. In the following experiment, therefore, litter mates of Bd and Od females were sterilized. A simple section of the end part of the uterus, cutting the communication between fallopian tube and uterus, was found to be insufficient to prevent pregnancy constantly. Hence, the end part of the uterus was fixed to the abdomen by means of a small thread, avoiding dislocation of the ovary as much as possible. In the evening the animals were put with the males and in the morning the vaginas were examined for the presence of a vaginal plug. The object of this method, to procure a greater number of copulations, was not attained, as after a few months

TABLE 3.—*Mammary tumor incidence in susceptible hybrids force-bred with males with and without the agent, and in their foster daughters*

Hybrids litter mates force-bred 10 times, then bred	Total number	Average number of litters	With mammary tumors			Nontumor-bearing—average age of death in days	Foster-daughters ♀ Odz				
			Number	Percent	Average age with tumor in days		Total number	With mammary tumors			Nontumor-bearing—average age at death in days
								Number	Percent	Average age with tumors in days	
♀ Od:											
With ♂d (with agent)	46	12	12	26	659	682	141	12	9	662	713
With ♂020 (agent-free)	48	12	12	25	709	740	108	5	5	683	726
♀ Bd:											
With ♂d (with agent)	62	11	6	10	623	703	70	3	4	683	700
With ♂B (agent-free)	32	12	0	0	----	709	40	3	8	753	723
With ♂020 (agent-free)	71	11	8	11	674	722	58	3	5	674	722
With ♂Bd (agent-free)	29	15	4	14	745	693	----	----	----	----	----

the cycle is arrested because in the ovarian sac liquid continually accumulates, finally leading to serious interference with ovarian function. The results are recorded in table 4. Copulation with males with the agent did not lead to the development of mammary tumors.

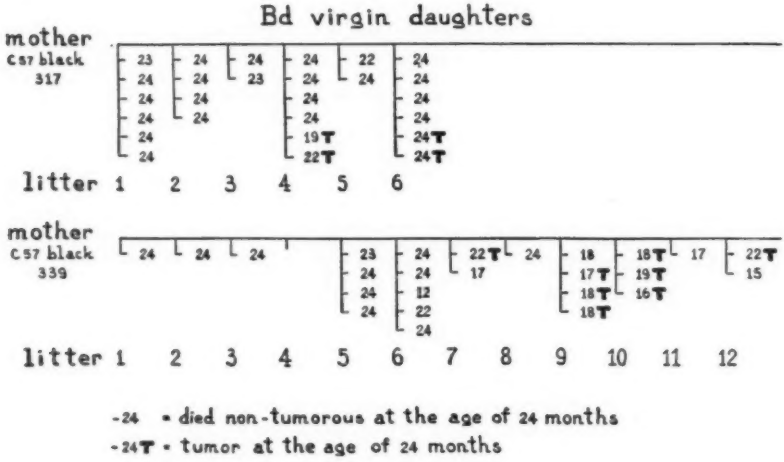
TABLE 4.—*Mammary tumor incidence in sterilized hybrids mated with males with and without the agent*

Sterilized hybrids	Number	Number of pseudo-pregnancy	With mammary tumors		Nontumor-bearing—average age at death in days
			Number	Tumor-age in days	
♀ Bd mated:					
With ♂d	11	6	0	----------	680
With ♂B	7	7	0	----------	651
Sterilized ♀ Od mated:					
With ♂d	11	----------	1	846	776
With ♂020	11	----------	0	----------	825

V. Analysis of the Mammary Tumor Incidence in Untreated Bd Hybrids

If, in copulation, the agent is transmitted with the sperm, either the mother or the young *in utero* may be "infected." If after infection of the mother the agent is transmitted to the young by the milk, then in certain litters practically all the sisters can be expected to have a mammary tumor. Further, it is to be expected that in this case an accumulation of mammary tumors will take place in the later litters.

With the aim of testing these hypotheses an analysis was made of the tumors developing in the F_1 hybrids from mating C 57 black females to dilute-brown males from our colony (table 5). A total of 43 mammary tumors has been observed in these Bd hybrids in the last few years. A total of 240 sisters from these 43 hybrids was kept untreated, either as virgins or as breeders, for the entire period of their lives, leaving 283 animals that could be used for analysis. These 283 hybrids were bred from 22 C 57 black mothers. In our colony mammary tumors in C 57 black females have not been noted in the last few years and also in these 22 C 57 black mothers no mammary tumors were found. Of the 43 tumors in hybrids, 14 were solitary mammary tumors, occurring in different litters, whereas their 143 sisters had no mammary tumors. In the 35 descendants from 2 B mothers there were 2 mammary tumors in the daughters of each mother, and in 34 of 3 mothers in each case 3 tumors. With one exception these mammary tumors occurred in different litters. In the daughters of the last 3 mothers the picture was different. An accumulation of mammary tumors occurred in the later litters. The distribution of the tumors in the hybrids of the last 2 mothers (No. 317, No. 339) is outlined in text-figure 1. All the daughters of these 2 mothers were kept as virgins. The accumulation of mammary tumors in the later litters points to the probability that in these cases the agent had been transmitted to the young by the father *via* the mother.


Bd virgin daughters
mother
C57 black
317
litter 1 2 3 4 5 6
mother
C57 black
339
litter 1 2 3 4 5 6 7 8 9 10 11 12
-24 = died non-tumorous at the age of 24 months
-24T = tumor at the age of 24 months

TEXT-FIGURE 1.

Morphology of the mammary tumors.—Survey of a large group of mammary cancers shows that the morphology does not follow a definite pattern. There are differences not only between the various forms of cancer but also one and the same cancer frequently presents different pictures. Recently Dunn (*15*) and Andervont and Dunn (*6*, *16*) have carefully weighed the possibilities of a classification of mammary tumors. We can confirm their conclusions from our own data: no rigid classification is possible and no great significance can be ascribed to the morphology of an individual tumor. Nevertheless, it is striking that in comparing a great many tumors in animals with and without mammary tumor agent and having the same constitution, the group without tumor agent reveals a greater number of tumors showing some unusual features. For the problem under discussion, namely, that of whether the development of the tumors found in the Bd females is due to the influence of the agent acquired by the father, the morphology of the tumors is of little assistance and has only descriptive value.

Tumor age.—As appears from table 5, the tumor age in a given case is not characteristic. On comparison, however, of the mean tumor age of the animals from the first three litters with that of the animals for the later litters, it appears that the tumor age in the first case is significantly higher (727 days) than that in the later litters (602 days). In this comparative investigation, owing to the small number of animals, no account was taken of whether the animals were kept as virgins or as force-breeders. It is well known that pregnancy lowers the mean tumor age in the Bd hybrids with the agent (*17*). Most of the animals with tumors from the first litters were kept as force-breeders, whereas most animals from the later litters were kept as virgins. Presumably, therefore, the difference in tumor age between the animals from the first few, and from the later litters is even greater than has been indicated. This

TABLE 5.—*Mammary tumors in Bd-hybrids*

Tumor No.	Case No. of C57 black mothers bred with ♂ d	Total number of Bd-daughters untreated	Number of Bd-daughters with mammary tumors	Kept as—	Tumor age in days	Litter in which mice with tumor occurred	Morphology of the mammary tumor
1	331	26	1	Virgin	692	2d	Acinar adenocarcinoma.
2	143	15	1	Force-bred	867	6th	Tubular adenocarcinoma solidum
3	195	14	1	do	885	3d	Papillary cystic adenocarcinoma.
4	247	13	1	do	893	2d	Carcinoma solidum.
5	261	13	1	do	840	2d	Fibroadenoma.
6	142	13	1	Virgin	755	3d	Adenocarcinoma.
7	992	12	1	Breeder	598	4th	Adenoacanthoma.
8	1, 338	11	1	Force-bred	762	4th	Papillary tubular adenocarcinoma.
9	268	11	1	do	800	2d	Epidermoid carcinoma.
10	274	8	1	do	682	1st	Papillary tubular adenocarcinoma.
11	197	7	1	do	552	2d	Adenoacanthoma.
12	3, 598	6	1	do	834	1st	Fibroadenoma.
13	325	5	1	do	804	1st	do.
14	990	3	1	Virgin	667	4th	Acinar adenocarcinoma.
15	237	28	2	Virgin (a)	721	2d	Adenocarcinoma solidum.
16				Virgin (b)	662	7th	Acinar adenocarcinoma.
17	296	7	2	Force-bred (a)	641	1st	Papillary cystic adenocarcinoma.
18				Force-bred (b)	721	1st	Fibrosarcoma.
19	298	13	3	Force-bred (a)	619	1st	Adenocarcinoma solidum.
20				Force-bred (b)	627	4th	Papillary cystic adenocarcinoma.
21				Force-bred (c)	731	4th	Acinar adenocarcinoma.
22	337	10	3	Force-bred (a)	565	1st	Papillary tubular adenocarcinoma.
23				Force-bred (b)	805	1st	Adenocarcinoma solidum.
24				Force-bred (c)	811	1st	Carcinosarcoma.
25	272	11	3	Force-bred (a)	824	1st	Adenoacanthoma.
26				Force-bred (b)	451	3d	Adenocarcinoma solidum.
27				Force-bred (c)	641	3d	Adenocarcinoma.

TABLE 5.—*Mammary tumors in Bd-hybrids*

Tumor No.	Case No. of C57 black mothers bred with ♂ d	Total number of Bd-daughters untreated	Number of Bd-daughters with mammary tumors	Kept as—	Tumor age in days	Litter in which mice with tumor occurred	Morphology of the mammary tumor
28	1, 324	6	4	Force-bred (a)	403	4th	Acinar adenocarcinoma.
29				Pseudopregnancy (b)	398	7th	do.
30				Pseudopregnancy (c)	469	7th	do.
31				Virgin (d)	635	7th	do.
32	317	26	4	Virgin (a)	568	4th	do.
33				Virgin (b)	663	4th	do.
34				Virgin (c)	720	6th	do.
35				Virgin (d)	622	6th	do.
36	339	25	8	Virgin (a)	673	7th	Sarcoma.
37				Virgin (b)	501	9th	Acinar adenocarcinoma.
38				Virgin (c)	543	9th	do.
39				Virgin (d)	494	9th	do.
40				Virgin (e)	535	10th	do.
41				Virgin (f)	573	10th	do.
42				Virgin (g)	466	10th	Adenocarcinoma solidum.
43				Virgin (h)	671	12th	do.
Total	22	283	43				

finding is in agreement with the experiences from other strains, in which it was also found that the tumors presumably free of the agent, develop at a later age.

Test for the presence of the mammary tumor agent in Bd hybrids with tumors.—Tumor No. 4 (see table 5): this mouse was bred with an 020 male without the agent and had had 17 litters. The 1–13 litters were discarded immediately after birth. To the 14–17 litters were added 2 foster daughters Odz each. None of these 8 foster daughters developed a mammary tumor: they died at an average age of 743 days. The blood of the Bd hybrid was tested; 0.2 ml. was injected intraperitoneally into each of 2 Odz females. Both died without tumors at the ages of 523 and 593 days. It may, therefore, be concluded that the bearer of this mammary tumor did not harbor the mammary tumor agent.

Tumor No. 7: this mouse was bred with a d male and had had 7 litters with 21 daughters. All were kept and force-bred with their brothers. Only one daughter from the 3d litter developed an undifferentiated carcinoma at the age of 454 days. The others died without tumors at an average age of 638 days. Therefore no mammary tumor agent was present.

Tumor No. 17: this mouse was force-bred 13 times with a d male. To the 14 and 15 litters were added 2 new-born Odz females each. These 4 foster daughters died without tumors at an average age of 694 days.

Tumor No. 25: this mouse was force-bred with an 020 male. To the 15th–17th litters were added 2 new-born Odz females each. All 6 foster daughters died without tumors at the average age of 789 days.

Tumor No. 27: this mouse was force-bred 12 times with a d male. Three foster daughters, Odz, were added in the 13–14 litters; all died without a tumor at the average age of 666 days.

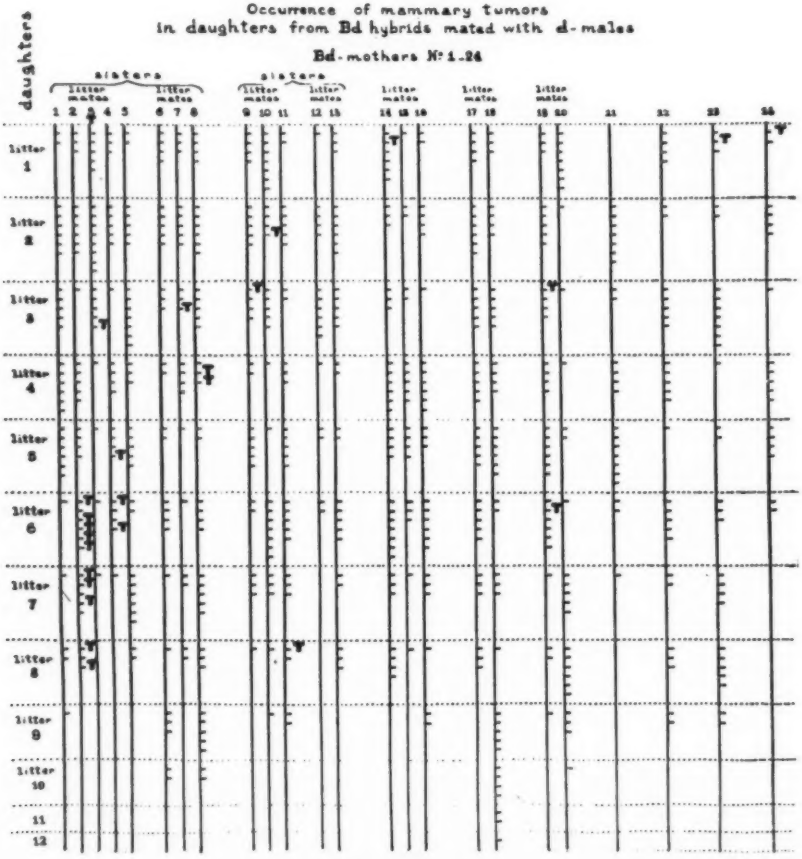
The five Bd hybrids with mammary tumors tested did not harbor the mammary tumor agent. All these hybrids were born in the first four litters of their B mothers. None of the bearers of mammary tumors from later litters, which presumably contained the agent were tested. *The analysis of this data suggests* that some C57 black mothers, by copulation with the dilute-brown male, acquired a sufficient quantity of agent to be transmitted with the milk to the daughters of the later litters. This explains the accumulation of mammary tumors in the daughters of the last litters. In this group also a number of mammary tumors is found distributed over several litters. A few of these tumors from the first litters have developed in the absence of the mammary tumor agent.

VI. Transmission of the Agent by the Male Parent

Analysis of the incidence of the mammary tumors in the Bd hybrids has suggested the probability that transmission of the agent by the male parent is possible. Only a limited number of animals was available for the investigation, these hybrids being usually needed for other experi-

ments. A special experiment was, therefore, indicated in which all descendants were to be kept untreated for the duration of their lives.

Analysis V had shown that special attention should be paid to the later litters. Accordingly Bd hybrids were used for the experiment, these latter usually producing a greater number of litters than the animals from pure strains. The percentage of spontaneous mammary tumors is very low in these Bd hybrids, although they are highly susceptible to the mammary tumor agent. Twenty-four Bd hybrids were bred with d males. Of the 24 Bd mothers only 1 developed a mammary tumor at the age of 598 days; the others died without tumors at the average age of 766 days. All daughters—a total of 674—were 3 times force-bred with their brothers and then kept till the end of their lives. Of the 674 daughters only 25 had mammary tumors at the average age of 591 days; the others died without tumors at the average age of 699 days. The distribution of these 25 mammary tumors is given in text-figure 2. In this dia-



TEXT-FIGURE 2.

gram each daughter is indicated by a small line, the letter T showing the daughter to have had a mammary cancer. Study of this diagram reveals that part of these mammary tumors are distributed at random among the mothers and the litters. Two groups of mammary cancer, however, are exceptions. The daughters from litters 6–8 from mother No. 2 nearly all show mammary cancer. The same is found in the daughters of mother No. 4 from litters 5 and 6. The daughters from the first litters died without tumors. This phenomenon should be interpreted as follows: these 2 mothers, after the 5th copulation, had acquired a sufficient quantity of the mammary tumor agent to enable them to transmit it to the daughters with the milk. If the mother has been infected then the appearance of mammary cancer in all or practically all daughters can of course be expected. Conversely, the appearance of a single mammary cancer among a great number of sisters indicates that in this case the agent in the mother's milk cannot have played a role.

In table 6 a survey arranged according to family is given of the morphology of the 25 mammary tumors in the Bd×d females. This table shows that the mammary tumors of mothers No. 2 and No. 4, with one exception, can all be classified as typical acinar adenocarcinomas. The morphological picture of these tumors is similar to that usually seen in tumors with the agent. Among the tumors in the daughters of other mothers various morphological patterns are found. The average tumor age of the mammary tumors in the daughters of mothers No. 2 and No. 4 is 550 days; that of all the other tumors combined, 637 days. Here also the repeatedly encountered phenomenon is found of the development at

TABLE 6.—*Morphology of the mammary tumors in Bd×d females with tumor-age in days*

Bd mother No. (See text-fig. 2)	
2	*a.* epidermoid carcinoma (460). *b.* acinar adenocarcinoma (710). *c.* acinar adenocarcinoma (545). *d.* acinar adenocarcinoma (573). *e.* acinar adenocarcinoma (651). *f.* acinar adenocarcinoma (578). *g.* acinar adenocarcinoma (680). *h.* acinar adenocarcinoma (330). *i.* acinar adenocarcinoma (500). *j.* acinar adenocarcinoma (476).
3	*a.* carcinoma solidum (454).
4	*a.* acinar adenocarcinoma (599). *b.* acinar adenocarcinoma (460). *c.* acinar adenocarcinoma (582).
7	*a.* cystic tubular adenocarcinoma (567).
8	*a.* carcinoma solidum (616). *b.* tubular adenocarcinoma (700).
9	*a.* carcinosarcoma (652).
10	*a.* epidermoid carcinoma (523).
11	*a.* carcinosarcoma (656).
14	*a.* acinar adenocarcinoma (772).
19	*a.* carcinoma solidum planocellular (574). *b.* undifferentiated carcinoma (740).
23	*a.* adenoacanthoma (554).
24	*a.* cystadenoacanthoma (836).

an earlier age of tumors with the agent compared to those presumably arising without the agent. None of the tumors was tested for the presence of the agent. One of the mothers (No. 3) had an adenoacanthoma; of the 22 daughters of this mouse only one had a mammary cancer, an undifferentiated form, at the age of 454 days, from which it may be inferred that this mother had no agent or at any rate that she had not transmitted it to her daughters in the milk.

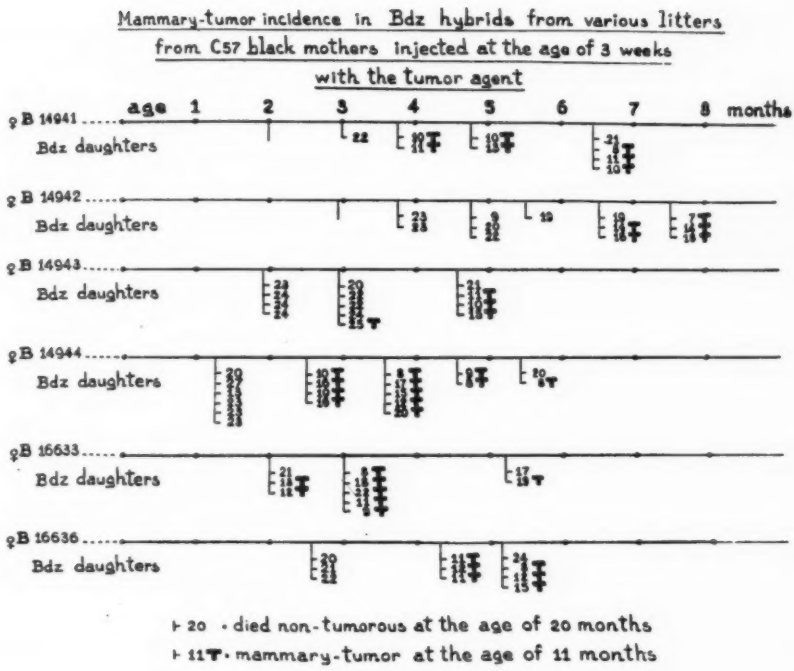
Conclusion.—This experiment suggests that transmission of the agent by the male parent is possible, but seldom occurs. The mother is infected and even if she herself does not develop a mammary cancer, she can transmit the agent to the daughters in the milk.

VII. Duration of Latency Period Following Infection With the Mammary Tumor Agent

Experiments V and VI have demonstrated that transmission of the agent by the male parent is possible in the sense of the mother acquiring the agent and transmitting it to the young in the milk. In both experiments it was remarkable that mammary tumors in the daughters only appeared in the later litters. Repeated copulations may be required for transmission to the mother of a quantity of the agent sufficient to produce the necessary concentration in the milk. Another supposition is that a single copulation is adequate, but that the propagation of the virus requires time. In order to investigate these two possibilities C57-black females at 3 weeks of age were injected intraperitoneally with tumor extract from a DBA female. The C57-black females were then mated with dz males without the agent and all Bdz daughters were kept. They were force-bred with their brothers for a year and afterward observed for the development of mammary tumors. The tumor frequency in these Bdz daughters has been summarized in text-figure 3. The results of these experiments appear to be in fairly close agreement as regards the occurrence of mammary tumors only in the daughters of the later litters, in spite of the fact that infection of the mother had taken place at the age of 3 weeks. The second case, ♀ B 14942, is especially remarkable. The first litter contained only males. The daughters from litters 2–4 died without tumors; daughters having mammary tumors were not observed until litters 5–6. The implications of these findings will not be discussed here, as the problem is being investigated further. These results reveal with sufficient clearness that infection of the mother by the father can lead in certain cases to such an accumulation of mammary tumors in the later litters. The results of the experiments also serve to demonstrate that a single copulation may infect the mother.

VIII. Comparison of the Mammary Tumor Incidence in Hybrids From Fathers With and Without the Agent

The results of the previous experiments have shown that a transmission of the agent at copulation is possible in the sense of the mother acquiring the agent and—without developing a mammary tumor herself—trans-


Mammary-tumor incidence in Bdz hybrids from various litters
from C57 black mothers injected at the age of 3 weeks
with the tumor agent
age 1 2 3 4 5 6 7 8 months
♀B 14941
Bdz daughters
♀B 14942
Bdz daughters
♀B 14943
Bdz daughters
♀B 14944
Bdz daughters
♀B 15633
Bdz daughters
♀B 15636
Bdz daughters
⊢ 20 · died non-tumorous at the age of 20 months
⊢ 11T · mammary-tumor at the age of 11 months

TEXT-FIGURE 3.

mitting it to her daughters. In the strains used for the experiments this transmission was shown to manifest itself only in the daughters of the later litters. It was found, moreover, that this transmission from father to daughter *via* the mother cannot be demonstrated in all cases. A transmission being possible, the percentage of tumors must vary in the hybrids according to whether the father was with or without the agent. Simple comparison of the percentage of tumors alone cannot be conclusive. If, for instance, the hybrids from a father with the agent come from the first three litters, where no transmission can be demonstrated, there will be no difference demonstrable between the percentage of tumors in these animals and that in hybrids from a father without agent. If, on the other hand, the group of hybrids includes those from later litters in which transmission has occurred, a difference in tumor percentage can be expected. In the following table (7*A*) only a comparison of the first three litters of the Bd hybrids (father with agent) and those of the Bdz hybrids (father without agent) can be presented. The number of hybrids from the later litters is too small to exclude the factor of chance. No mammary tumors were found, either in the Bd virgins, or in the Bdz virgins from the first three litters, whereas they were found in both kinds of hybrids after force-breeding. The difference in tumor incidence is not significant.

TABLE 7.—*Mammary tumor incidence in hybrids from fathers with and without the agent*

	Total number	With mammary tumors			Nontumor-bearing	
		Number	Percent	Average age in days	Number	Average age at death
A. ♀ Bd (from 1st–3d litter) (father with agent):						
Virgins	89	0	------	------	89	733
Force-bred	88	11	12	755	108	725
♀ Bdz (father without agent):						
Virgins	63	0	------	------	63	677
Force-bred	81	4	5	528	77	657
B. ♀ Bd×d (from 1st–3d litter) (father with agent): Force-bred	82	3	3	689	79	735
♀ Bd×dz (father without agent): Force-bred	115	0	------	------	115	688

Table 7 *B* gives the same comparison between ♀ Bd×d (father with agent) and ♀ Bd×dz (father without agent). Here again, only the animals from the first three litters, from the Bd mothers as well as from the daughters of the back-cross with a d father, have been included. There is no difference in mammary tumor incidence between these two groups either. The conclusion appears justified that the mammary tumors in the groups *A* and *B* in table 7 arise without the intervention of the father's agent. From this it follows that the concept of an infection *in utero* by the sperm as the cause of the development of these tumors in the daughters, a possibility also tentatively suggested by Andervont, is not supported by the results of these experiments. A comparison of the tumor incidence between virgins and force-bred animals in group *A* in table 7 confirms the phenomenon, repeatedly observed, that pregnancy is the decisive factor in the development of mammary tumors in animals without the agent. The virgin females have no mammary tumors.

DISCUSSION OF RESULTS

The starting point of this investigation was the observation that in hybrid mice procured by mating low-cancer strain females to high-cancer males the number of mammary tumors noted was always higher than that in the maternal strain. Various experiments were carried out to test the hypothesis of the transmission of the mammary tumor agent with the sperm. A premise was the demonstration of the agent in the sperm. In the first experiment of the investigation it was further shown that the agent in the sperm, following its intraperitoneal injection into the mother, is transmitted to the young with the milk. Two possibilities are to be considered in the transmission of the agent with the sperm at copulation: 1) The agent can "infect" the mother, resulting in either development or nondevelopment of the mammary cancer in the mother. The agent being transmitted to the mother's milk, it follows that in "infection" of the mother the daughters will develop a mammary cancer if the other conditions—proper genetic constitution and adequate hormonal stimulation—have been fulfilled. 2) The other possibility is that the young them-

selves are "infected" *in utero*, either with or without simultaneous "infection" of the mother.

In experiment II female litter mates of the low-cancer 020 Leeuwenhoek-Huis strain and females from the Dz line without the agent were force-bred with d males with agent and with dz males without agent. The mammary tumor incidence in both groups was the same, leading to the conclusion that in these strains "infection" of the mother in a manner giving rise to later development of a mammary tumor in the mother is not possible. The same results were obtained with susceptible hybrids F_1 (♀ 020×♂d) and F_1 (♀ B×♂d), in which equal numbers of litter mates were mated with males with and without the agent. The percentage of mammary cancer showed no significant difference between the various groups, in spite of the fact that these animals had a greater number of litters and accordingly also many copulations. The assumption is that in copulation there is insufficient absorption of sperm for provoking the development of a mammary cancer in the mother, as is seen to occur following intraperitoneal injection of sperm. Multiple experiences have shown that owing to the prolonged period of latency, administration of the mammary tumor agent in advanced age fails to bring about the development of mammary cancer, even in susceptible animals. Assuming that with each copulation an inadequate quantity of agent is transmitted to the female, it is obvious that only the first three copulations may be the cause of a mammary cancer appearing at a later date. The other copulations, even when transmission of the agent occurs, cannot affect the mother. For the daughters of these mothers, however, the situation is entirely different. If the mothers have been infected with the agent in advanced age, even if they themselves have no mammary cancer, there is still the possibility of the agent being transmitted to the young with the milk.

In experiment III, therefore, after the 10th pregnancy from fathers with or without the agent, susceptible hybrids were given as foster daughters to be fed by these mothers. It was found that the mammary tumor percentage in these foster daughters showed no difference in the different groups of mothers. Hence, this percentage is independent of the fact whether these mothers had copulated with males with, or those without, the agent. The conclusion to be drawn from this experiment is, therefore, that in copulation with males with the agent the mothers are not, or not sufficiently, "infected" to enable them to transmit the agent with the milk to their foster daughters. Analysis of the mammary tumor incidence in Bd hybrids from our colony, however, demonstrated that in a few cases accumulation of mammary tumors occurred in later litters. This finding permits the presumption that the agent played a rôle in the development of these tumors. This led to experiment VI, in which a group of Bd females were back-crossed with d males with agent, keeping all 674 daughters. In the daughters of 2 mothers an accumulation of mammary tumors was likewise found in the later litters; therefore, it may be assumed that these mammary tumors were due to the agent from the mother's milk. The morphological picture of this group of tumors sup-

ports this concept. These mammary tumors have not been tested for the presence of the agent; the father's sperm is considered to be the source. The results of a control experiment (VII), in which B mothers were injected with tumor extract, also plead for the correctness of this assumption. Here also, a mammary cancer was observed only in the daughters from the later litters, those from the first few litters showing no tumor. An "infection" of the mothers with the agent from the sperm can, therefore, be presumed, this infection giving rise to the development of mammary cancer in their daughters. In experiment III, comparison of the groups in which the mothers copulated with males with agent, and those in which the mothers copulated with males without agent, showed no difference in tumor incidence between the foster daughters of these two groups. This finding remains unexplained. In addition to the mammary tumors in which the presence of the agent may be assumed, there are a number of mammary cancers distributed over the various litters. Transmission of the agent with the mother's milk cannot be assumed as a cause for the development of these tumors. Comparative analysis of these mammary tumors demonstrates that they cannot be related to the presence of the agent in the father. Presumably mammary tumors are capable of arising also in the absence of the agent. Pregnancy must be considered as a causative factor in their development.

SUMMARY

Studies on the transmission of the mammary tumor agent by the male parent, using hybrids of the dilute-brown and the C57 black and 020 Leeuwenhoek-Huis strains, yielded the following results:

1. The sperm of males of the dilute-brown strain contains the mammary tumor agent. This agent from the sperm, following intraperitoneal injection, can be transmitted with the milk to the daughters of susceptible hybrids.

2. Prolonged force-breeding of agent-free female mice with males with, and those without, agent does not result in a rise of the tumor percentage in the group bred with males with agent.

3. Addition of susceptible foster daughters following prolonged force-breeding gives no grounds for the assumption of a transmission of the agent to the females bred with males with agent.

4. Analysis of the mammary tumor incidence in untreated Bd hybrids resulted, in a few cases, in an accumulation of mammary tumors in the later litters. Transmission of the agent from the father to the daughters *via* the mother's milk is assumed to be the causative factor.

5. Following back-crossing of Bd females with DBA males with agent an accumulation of tumors in the later litters occurred in a few families. Of 674 daughters, 25 had a mammary cancer. Analysis of these mammary tumors makes it probable that the mother is "infected" by the father; even when the mother herself does not develop a mammary cancer the agent can be transmitted to the daughters with the milk.

6. Following injection of tumor extract with agent to C57 black mothers

at an early age the agent is only transmitted to the daughters in the later litters.

7. Comparison of the mammary tumor incidence in hybrids from fathers with and without the agent gives no support to the concept that infection of the young *in utero* by the sperm of the father is possible. It may be assumed that mammary tumors can also arise in the absence of the agent. Pregnancy must be considered as a causative factor for these tumors.

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THE EFFECT OF LOCAL INJECTIONS OF ADRENALIN ON EPIDERMAL CARCINOGENESIS IN THE MOUSE [1]

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Orr (*9, 10*) reported that when skin tumors were produced in the mouse by repeated applications of tar or 1,2,5,6-dibenzanthracene, the rate at which tumors appeared could be increased by injecting adrenalin subcutaneously beneath the painted area immediately before each application of carcinogen. The increase in the rate of tumor production was apparent from the beginning, more tumors appearing in the animals given adrenalin than in the controls.

If tumors are induced, not by repeated applications of carcinogen such as Orr used, but by painting once only with carcinogen and then repeatedly with croton oil, carcinogenesis can be divided into two "stages" (*2, 3, 8*); in the first, a dose of carcinogen too small to produce more than an occasional papilloma changes the skin so that in the second the noncarcinogenic substance croton oil is able to make papillomata manifest in the prepared skin. The first of these stages will be called "initiation," and the second "promotion" (*7*). If a 4-week interval be allowed between the application of carcinogen and the first application of croton oil, the stages can be separated, for initiation is probably complete by the time the carcinogen has disappeared from the skin, and though estimates differ, the carcinogen probably goes in less than 4 weeks (*1, 3, 6, 16*).

The effect of injections of adrenalin on these stages was examined. The stages were separated by applying 9,10-dimethyl-1,2-benzanthracene once, and after a 4-week interval beginning applications of croton oil. To determine its effect on initiation, adrenalin was injected while the carcinogen was still present in the skin; to determine its effect on promotion, an injection was given each time croton oil was applied. The injections given at the time of initiation cannot have affected promotion, for it will be shown that the action of adrenalin is fleeting, and the last injection was given 2½ weeks before the first application of croton oil. Nor can the injections given during promotion have affected initiation, for the first was not until 4 weeks after the application of the carcinogen,

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and by that time initiation was probably complete. The effect of injections of adrenalin on carcinogenesis by repeated applications of 1,2,5,6-dibenzanthracene was also determined, Orr's experiment being repeated on a small scale.

Rous (*13*) and Rous and Drury (*14*) showed that when mice were dyed red by injecting phenol red intraperitoneally, ischemic areas turned yellow. Advantage was taken of this observation to determine how long the local effect produced by injections of adrenalin persisted.

METHOD

Virgin female mice of the M. R. C. Swiss strain were used. They were fed on the rat cake supplied by the North Eastern Agricultural Co-operative Society of Aberdeen, both food and water being given *ad libitum*.

The painted area was a square in the interscapular region kept free of hair by clipping with scissors. The 9,10-dimethyl-1,2-benzanthracene was applied as a 1.5 percent solution in mineral oil; the 1,2,5,6-dibenzanthracene as a saturated solution in acetone; and the croton oil as a 5-percent solution of Ol. Croton. B. P. (Boots) in mineral oil. Two solutions of adrenalin were used. Adrenalin "A" was Solution of Adrenaline B. P. (Martindale) diluted to a strength of 1/10,000 with normal saline. As injected, this solution contained 0.05 percent of chlorbutol. Adrenalin "B" was a 1/10,000 solution of pure adrenalin (B. D. H.) made up as the hydrochloride after the addition of the calculated amount of acid. Both solutions were prepared immediately before use. The injections were given by Orr's method. A fine needle was introduced outside the painted area and passed subcutaneously so that the solution was delivered in the center of the painted area as near to the skin as possible, though not intradermally. In the 1,2,5,6-dibenzanthracene experiment each injection was of 0.05 ml.; and in the other experiments, of 0.1 ml. The solution of phenol red (B. D. H.) was prepared and injected as described by Rous (*13*).

Each mouse was examined twice a week, and the position of the tumors charted. The results have been calculated from these charts. When tumors are produced by carcinogen and croton oil, some regress, some persist, and some become malignant (*15*). It is difficult to decide whether or not those tumors that regress very shortly after their appearance are true papillomata. Perhaps, as Cramer and Stowell (*4, 5*) suggested, they are but patches of dermal edema. In this paper an arbitrary rule has been adopted, and only those tumors that lasted more than 4 weeks have been counted. All seemed to be true papillomata, and except in the 1,2,5,6-dibenzanthracene experiment none became malignant. Only the appearance of tumors is recorded. That some regressed after having been present more than 4 weeks is ignored. Again, only those animals that survived until the end of the experiment have been considered. Such a simplification is statistically permissible if the animals with tumors are no more likely to die than those without. In these experiments the assumption was justified. The tables show the total number of tumors

that appeared in the animals surviving until the end of the experiment, and the number of tumor-bearing animals. Also shown, to facilitate comparison, is the average number of tumors per tumor-bearing mouse. Time is measured from the first application of croton oil. The "latent period" of a tumor is the time from the first application of croton oil to the appearance of that tumor. It should be noted that this definition differs from that usually given. Time is measured from the first application of croton oil, not from the first application of carcinogen. The "average latent period" is the mean of the latent periods for a group.

DYE EXPERIMENTS

In 20 mice adrenalin was injected under the clipped area by Orr's technique, and phenol red given intraperitoneally at varying intervals afterwards. The experiment was repeated several times in each animal. In no case was the yellow color indicating ischemia demonstrable for more than 2 hours after the injection of adrenalin, and in few for more than 1 hour.

ADRENALIN GIVEN AT THE TIME OF INITIATION

Three groups of mice were painted once with a large dose of 9,10-dimethyl-1, 2-benzanthracene. Four weeks later applications of croton oil were begun, and were continued twice a week throughout the experiment. Each animal given adrenalin received four injections, the first immediately before the painting with carcinogen, and the others at half weekly intervals thereafter. The first group, of 50 mice, was given injections of adrenalin "B"; the second, of 25 mice, injections of adrenalin "A"; and the third, of 25 mice, no injections at all.

Table 1 shows the results. There was little difference between the three groups, the values for the control lying between those for the two adrenalin groups. More tumors appeared after week 30 in the adrenalin groups than in the control, but the difference was small.

ADRENALIN GIVEN AT THE TIME OF PROMOTION

Three groups of 25 mice were painted once with a large dose of 9,10-dimethyl-1,2-benzanthracene. Four weeks later applications of croton oil were begun, and were continued twice a week throughout the experiment. The first group was given injections of adrenalin "A," the second of normal saline, while the third received no injections. Each mouse injected was given an injection immediately before each application of croton oil; that is, twice a week from the first application of croton oil until the end of the experiment.

Table 2 records the results. There was little difference between the three groups until after week 30, when more tumors appeared in the adrenalin group than in the controls. The significance of this late difference is, again, doubtful.

TABLE 1.—*Adrenalin given during the initiating stage of carcinogenesis by a single application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil*

	Group given adrenalin "B"[2] (36 survivors)			Group given adrenalin "A"[2] (19 survivors)			Group given no injections (16 survivors)		
	Total number of tumors[1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse	Total number of tumors[1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse	Total number of tumors[1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse
Number of weeks after first application of croton oil:									
5	3	2	1.5	5	1	5.0			
8	80	15	5.3	68	14	4.9	52	8	6.5
11	146	24	6.1	139	17	8.2	93	10	9.3
14	188	28	6.7	168	17	9.9	133	15	8.9
17	240	30	8.0	203	17	11.9	146	15	9.7
20	307	33	9.3	216	18	12.0	176	16	11.0
23	350	34	10.3	231	18	12.8	183	16	11.4
26	364	36	10.1	238	18	13.2	190	16	11.9
29	371	36	10.3	241	18	13.4	193	16	12.1
32	385	36	10.7	250	18	13.9	198	16	12.4
35	431	36	12.0	263	18	14.6	205	16	12.8
38	449	36	12.5	273	18	15.2	211	16	13.2
Average latent period in weeks:									
All tumors		17.9			15.0			14.6	
Tumors appearing before week 30		14.3			12.4			12.8	
Percentage of tumors, appearing after week 30		20			14			9	

[1] The tumors were all papillomata.
[2] Adrenalin "A" was Adrenalin (Martindale) and adrenalin "B" was Adrenalin (B. D. H.).

TABLE 2.—*Adrenalin given during the promoting stage of carcinogenesis by a single application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil*

	Group given adrenalin (16 survivors)			Group given saline (16 survivors)			Group given no injections (20 survivors)		
	Total number of tumors [1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse	Total number of tumors [1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse	Total number of tumors [1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse
Number of weeks after first application of croton oil:									
5									
8	3	1	3.0	5	1	5.0	3	2	1.5
11	10	9	1.1	15	8	1.9	10	4	2.5
14	30	10	3.0	26	9	2.9	33	9	3.1
17	49	10	4.9	42	9	4.7	60	11	5.5
20	58	12	4.8	47	9	5.2	73	15	4.9
23	72	12	6.0	62	9	6.9	89	16	5.6
26	81	14	5.8	67	11	6.1	101	16	6.3
29	88	14	6.3	78	12	6.5	111	16	6.9
32	106	14	7.6	91	13	7.0	113	16	7.1
35	128	16	8.0	103	13	7.9	121	16	7.6
38	146	16	9.1	111	13	8.5	126	17	7.4
Average latent period in weeks:									
All tumors		24.0			22.4			20.0	
Tumors appearing before week 30		18.3			17.8			18.1	
Percentage of tumors appearing after week 30		40			30			12	

[1] The tumors were all papillomata.

ADRENALIN GIVEN DURING CARCINOGENESIS BY REPEATED APPLICATIONS OF 1,2,5,6-DIBENZANTHRACENE

Orr's experiment was repeated. Three groups of mice were painted once a week with 1,2,5,6-dibenzanthracene. The first group was given an injection of adrenalin "A" immediately before each painting, the second an injection of normal saline, while the third received no injections.

The results are shown in table 3. No increase in the rate of papilloma formation is evident.

DISCUSSION

Orr's (*9*, *10*) report that adrenalin injected subcutaneously beneath the painted area during epidermal carcinogenesis in the mouse caused an immediate increase in the rate of tumor production has not been confirmed. Adrenalin was given during the initiating stage of carcinogenesis by a single application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil, during the promoting stage, and throughout carcinogenesis by repeated applications of 1,2,5,6-dibenzanthracene. In no case was there an immediate increase in the rate at which papillomata appeared. It should be remembered that Orr thought his results suggestive, not conclusive.

If carcinogenesis were modified at all by the adrenalin, the effect was delayed, more papillomata appearing after week 30 in the adrenalin groups than the controls. However, the differences were not great and their significance is doubtful.

Orr (*9*, *10*, *11*) suggested that adrenalin might produce its effect by causing ischemia, and reported that rendering the painted area ischemic by producing a subcutaneous fibrosis also caused an increase in the rate of tumor production. In the rabbit, a similar increase in the rate of papilloma formation can be produced by making the painted ear ischemic (*12*). It might be that the ischemia produced by the injections of adrenalin in these experiments was too slight to be effective.

SUMMARY

The effect of local subcutaneous injections of adrenalin on epidermal carcinogenesis in the mouse was reinvestigated. It could not be confirmed that the adrenalin causes an immediate increase in the rate of papilloma formation. If any modification of carcinogenesis were produced, it was a delayed effect, more papillomata appearing in the adrenalin groups after week 30, but the significance of this late effect is very doubtful.

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TABLE 3.—*Adrenalin given throughout carcinogenesis by repeated applications of 1,2,5,6-dibenzanthracene*

	Group given adrenalin (18 survivors)			Group given saline (22 survivors)			Group given no injections (21 survivors)		
	Total number of tumors [1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse	Total number of tumors [1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse	Total number of tumors [1]	Tumor-bearing mice	Average number of tumors per tumor-bearing mouse
Number of weeks after first application of carcinogen:									
5									
8									
11									
14				2	1	2.0	1	1	1.0
17				4	1	4.0	1	1	1.0
20				4	1	4.0	1	1	1.0
23	4	3	1.3	9	5	1.8	9	8	1.1
26	5	4	1.3	10	6	1.7	13	10	1.3
29	10	9	1.1	12	7	1.7	20	13	1.5
30	11	9	1.2	15	7	2.1	22	13	1.7
Average latent period in weeks	25.5			22.7			24.7		

[1] All the tumors were at first papillomata. Some became malignant.

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THE EFFECT OF ARTERIAL OCCLUSION ON EPIDERMAL CARCINOGENESIS IN THE RABBIT [1]

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As will be reported below, it was found that in the rabbit a lasting ischemia could be produced in one ear by tying the common carotid artery on that side. This paper describes the effect of this ischemia on epidermal carcinogenesis by repeated applications of 9,10-dimethyl-1,2-benzanthracene, the contralateral ear serving as a control.

METHOD

The rabbits were kept under standard conditions in single cages, and fed a mixed diet of greens, grain, and hay. No water was provided.

The right common carotid artery was exposed by making a longitudinal incision to the right of the midline, separating the strap muscles by blunt dissection, and carefully freeing it from the adjacent nerves. The artery was tied twice at points some inch apart, and divided between the ties.

The carcinogen was a 1.5 percent solution of 9,10-dimethyl-1,2-benzanthracene in mineral oil. It was applied to the whole inner surface of the ear.

The ears were examined once a week, and the position of the tumors traced onto celluloid with a grease pencil (*9*). From these tracings permanent records were made on squared paper. The tumors produced were papillomata of the usual types. Many small keratoses appeared that lasted only a short time, and were not considered true papillomas. An arbitrary rule has been adopted, and only those tumors lasting more than 4 weeks and showing some evidence of growth considered. The latent period of a tumor is taken as the time from the first application of carcinogen to its first appearance.

CHANGES PRODUCED IN THE EAR BY TYING ONE CAROTID ARTERY

When one carotid artery was tied the affected ear became slightly cyanotic, with marked dilatation of the veins. If both ears were heated, there was less reactive hyperemia on the affected side, and cyanosis be-

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came more apparent. Edema was not observed at any time. The changes persisted with little alteration for more than 2 years.

IMMEDIATE EFFECT OF ISCHEMIA ON CARCINOGENESIS

In five rabbits in which the right carotid artery had been exteriorized by the method of van Leersum (*2*), the artery became obstructed. As soon as the obstruction was certain, painting with 9,10-dimethyl-1,2-benzanthracene was begun and was continued twice a week throughout the experiment.

In a second group, the right carotid was tied in 6 rabbits, and half a week after the operation, applications of 9,10-dimethyl-1,2-benzanthracene were begun. In each rabbit the carcinogen was continued twice a week until the first papilloma appeared, from 8 to 14 applications being needed.

Table 1 records the results. The rate at which papillomata appeared was increased on the ischemic side. The difference was apparent in each rabbit.

TABLE 1.—*Immediate effect of ischemia on carcinogenesis by repeated applications of 9,10-dimethyl-1,2-benzanthracene*

	Carcinogen applied throughout (5 rabbits)		Carcinogen applied until appearance of 1st tumor (6 rabbits)	
	Tumors present [1]		Tumors present [1]	
	Ischemic ears	Nonischemic ears	Ischemic ears	Nonischemic ears
Number of weeks after first application of carcinogen:				
3	----------	----------	1	1
4	----------	----------	3	2
5	----------	----------	12	7
6	----------	----------	17	8
7	4	1	19	8
8	13	1	22	11
9	15	1	29	14
10	21	4	34	15
11	26	6	35	17
12	44	12	35	17
13	54	16	35	17
14	54	18	35	17
15	62	25	30	13
Average latent period (weeks)	11. 3	12. 6	7. 5	7. 3

[1] The tumors were all papillomata.

LATE EFFECT OF ISCHEMIA ON CARCINOGENESIS

In the group given carcinogen throughout so many tumors appeared that it became impossible to count them accurately, but in the group painted until the first papilloma appeared most of the tumors regressed before week 30. Three of the rabbits in which no tumors at all persisted were then painted again, 9,10-dimethyl-1,2-benzanthracene being applied

twice a week as before until a papilloma appeared. From three to five applications were needed.

Table 2 records the findings in these three animals. The tumors were all papillomata. Most regressed, but as some disappeared others appeared or reappeared, so that the number actually present at any time remained about the same. The rate at which this interchange took place is given by the number of papillomata appearing or reappearing per week. As the number present remained roughly constant, the number regressing must have been approximately equal to the number appearing. Both the rate of interchange and the number of papillomata present were greater on the ischemic side. The effect was apparent in each rabbit.

TABLE 2.—*Late effect of ischemia on carcinogenesis by repeated applications of 9,10-dimethyl-1,2-benzanthracene*

	Carcinogen reapplied after disappearance of first crop of tumors [1] (3 rabbits)	
	Tumors present [1]	
	Ischemic ears	Nonischemic ears
Number of weeks after first application of carcinogen: [2]		
30		
33		
36	11	3
39	23	7
42	25	9
45	26	8
48	28	5
51	23	4
54	21	4
72	14	4
75	15	5
78	15	5
81	14	7
84	19	2
87	18	2
90	20	2
93	23	2
96	19	4
Average number of tumors appearing or reappearing per week:		
Weeks 39–54	1.5	0.5
Weeks 78–96	.8	.4

[1] The tumors were all papillomata.
[2] Carcinogen was reappliedfrom week 30 until appearance of first tumor.

DISCUSSION

In the rabbit, when one ear was rendered ischemic by occluding the carotid artery, and both ears were painted repeatedly with 9,10-dimethyl-1,2-benzanthracene, the rate at which papillomata appeared was higher on the ischemic side. The difference was still apparent at the end of the experiment, more than a year after the last application of carcinogen.

With this finding can be compared Orr's report that when mice were

painted repeatedly with carcinogen, a similar increase in the rate at which tumors appeared could be produced by making the painted area ischemic (*6*, *7*). Some of his work was repeated, but could not be confirmed, no such increase being evident (*8*). This failure to confirm Orr's report might be due, not to the inability of ischemia to increase the rate of papilloma formation in the mouse, but to the failure of the method used to induce adequate ischemia.

That the number of tumors actually present eventually became more or less constant seems to be at variance with the report of Friedewald and Rous (*1*). However, the experiments are not comparable. Friedewald and Rous not only applied carcinogen, but also punched holes through the ears. In the rabbit, such punching greatly increases the number of tumors that appear (*3*, *4*, *5*, *10*), and introduces a factor not present in these experiments.

SUMMARY

In the rabbit, rendering the painted area ischemic increased the rate at which papillomata were produced by repeated applications of 9,10-dimethyl-1,2-benzanthracene.

Thanks are due to Dr. I. Berenblum for advice and to Mr. H. W. Wheal for technical assistance.

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THE INFLUENCE OF CORTISONE ACETATE UPON THE GROWTH OF A TRANSPLANTED RHABDOMYOSARCOMA IN C3H MICE [1] [2]

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Heilman and Kendall, in 1944 (*1*), made the initial study of the effects exerted by 17-hydroxy-11-dehydrocorticosterone upon the growth of a transplanted tumor. They reported complete resorption of a well-established lymphosarcoma of mice within 4 days, upon the daily administration of this adrenal steroid.

Since this initial study was made, the effectiveness of this hormone, cortisone, in controlling various types of experimental and chemical neoplasia has been repeatedly tested. Temporary regression of lymphoid tumors was reported soon after cortisone became available for clinical use (*2*), but invariably growth of the tumors was resumed upon the cessation of hormone therapy (*3*). Transplants of lymphosarcoma regressed in riboflavin-deficient mice when they were given cortisone (*4*); induced carcinomas of the skin were inhibited by the topical application of cortisone (*5*). Large amounts of the hormone retarded but did not suppress the growth of transplanted Walker-rat carcinomas (*6*). The influence of the hormone was tested upon the growth of a number of small-animal tumors by Sugiura and co-workers (*7*), with results that indicated inhibition but never complete suppression. Transmitted lymphatic leukemia in Akm mice was somewhat resolved by cortisone, but the survival time of such mice was only slightly prolonged (*8, 9*). More recently, a report emanating from our laboratory showed that experimental ependymomas, originally induced by methylcholanthrene and subsequently transplanted for a number of transplant generations, were definitely inhibited by the daily administration of 0.5 mg. of cortisone (*10*).

In view of the particular affinity that cortisone appeared to have for tissues of mesenchymal origin (*11–15*), we were prompted to test the effect of the hormone upon experimentally induced transplantable muscle tumors. Fortunately for our inquiry, a cross-striated muscle tumor had

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[2] With the technical assistance of Kathryn A. Woods.

been induced in C3H mice by a single injection of 0.5 mg. of 20-methylcholanthrene in 0.05 ml. of lard, filtered at 37° C. (*16*). Tumors developed in 57 of the 80 mice tested, and 8 of these tumors were pure rhabdomyosarcomas. Through the courtesy of Dr. Elizabeth Green of the Institute for Cancer Research in Philadelphia we were able to obtain this tumor, then in its sixty-seventh transplant generation. She sent, for study, sections of the original tumor. These sections (figs. 1 and 2) showed the cross striations and other cellular characteristics that identified the tumor as a true rhabdomyosarcoma. However, it should be stated that cross striations were not identified in any of the sections made of the tumors we obtained for our testing program.

EXPERIMENTAL METHOD

To test its growth in our strain of mice, tumor tissue from the original donor animal was removed as aseptically as possible, fragmented and transferred by trocar to subcutaneous pockets of 10 young adult male mice of our C3H strain. Implants grew well in 100 percent of the mice. They were visible on the fourth day, and well-circumscribed tumors were palpable on the seventh day. Thereafter growth was rapid, so that extensions into the groin and axilla, with metastasis to the regional lymph nodes, occurred. Survival time proved to be approximately 28 days.

Twenty male mice of the same age and strain were then implanted with viable fragments of the tumor. Ten of these mice were given cortisone intramuscularly at a rate of 50 mg. per kilogram of body weight, on the day of implantation and upon each successive day thereafter for 7 days. The remaining 10 mice were given intramuscular injections of a saline solution and served as controls.

Twenty male mice of the same age and strain were implanted with tumor fragments that were allowed to grow before cortisone therapy was instituted. On the seventh day after transfer these mice were arranged into two groups, equally distributed on the basis of the attained size of the tumors. Each mouse of one group was then given cortisone daily, intramuscularly, at a rate of 100 mg. per kilogram of body weight. Each mouse of the second group was given the same amount of a saline solution and served as a control. One mouse of the test group was killed on each of the second, third, and fourth days of cortisone administration, and sections of the tumors together with the integument and underlying tissues of the abdominal wall were excised and prepared for histologic study.

RESULTS

In our initial experiment, in which cortisone was given to test mice on the day of implantation and on each day thereafter, all tumors in all control mice grew well, and by the seventh day palpable masses, which measured 12 by 15 mm., had developed. Sections of these tumors (fig. 3) showed them to be comprised of discrete pleomorphic cells. Many cells were spindle shaped, with short cytoplasmic processes, but all were devoid of the cross striations that were characteristic of the tumors

seen after their induction (figs. 1 and 2). The nuclei were large and often vesicular, and the cytoplasm was restricted to narrow marginal zones. Marked contrasts in the growth of the implants in those mice that received the daily amounts of cortisone were obvious after the fourth day. By the seventh day after transfer, only small masses of tumor tissue measuring 4 to 5 mm. in diameter were palpable in all 10 animals that received cortisone. Cortisone, in the amounts given, had definitely inhibited the rate of growth of the implants. But upon withdrawal of the hormone, rates of growth comparable to those we had seen in control mice were observed, so that by the tenth day after transfer, tumors were obtained that were equal in size to those growing in control mice 7 days after implantation. Sections of tumor tissue removed and fixed on the seventh day showed that whereas we had restricted the growth of transplants in mice by giving cortisone and had induced an extensive necrosis throughout the tumor, small islands of viable tumor tissue remained to form foci from which rapid growth occurred upon withdrawal of the hormone.

In our second experiment, cortisone was withheld from mice bearing transplanted tumors for 7 days after transplantation; thus they had rapidly growing tumors before the course of hormone therapy was instituted.

The administration of cortisone, at a rate of 100 mg. per kilogram of body weight, exerted a marked effect upon the further growth of the tumors. They rapidly regressed and on the fourth day of therapy (11 days after transfer) they were approximately one fourth the size of the tumors growing in the untreated control animals. A series of cross sections of tumors taken from control and test animals at specified intervals after implantation and treatment is shown in figure 4. The contrast in size between the tumor of the untreated animal removed on the eleventh day (fig. 4*C*) and that removed from the animal receiving four daily injections of cortisone (fig. 4*F*) is obvious. Figures 5, 6, and 7 portray sections through test tumors and represent respectively the tumors in figure 4*D*, *E*, and *F*.

Tumors that were removed from control untreated mice 9 and 11 days after implantation were like that portrayed in figure 3. Mitotic figures were abundant. Tumor tissue had invaded the surrounding fat tissue of the host as well as the musculature of the abdominal wall. Focal areas of necrosis had developed in the central regions of the tumor where vascularity had failed to keep pace with the rapid growth of the tumor.

After the second injection of cortisone into mice bearing well-established tumors, on the ninth day after transfer, diffuse necrosis had occurred, so that necrotic material and cellular debris were widely distributed throughout the tumor (fig. 5). Nests of viable tumor cells persisted, and many of the tumor cells were phagocytic, engulfing fragments of the necrotic material. After the third and fourth injections of cortisone (figs. 6 and 7) the number of viable cells was less, the size of the tumor was reduced, and the extent of necrosis in proportion to viable tissue was

increased. Nuclei were compact and pyknotic, and the cytoplasmic zones were wider than before. Fibroblastic proliferation was obvious, and a resulting fibrosis was developing.

The usual toxic reactions hitherto seen in mice receiving such large amounts of cortisone developed in these animals on the second and third day. They ate less food and their body weights dropped rapidly from 20.9 gm. to 15 or 16 gm. It is presumed that their electrolyte balance was upset—that they had presumably sustained a loss of potassium and chloride and that they were in negative nitrogen balance. It is presumed, too, that they were hyperglycemic and that glycosuria had developed. These factors were undetermined, but the appearance of the animals led us to conclude that these well-known physiologic reactions to excessive amounts of cortisone had developed.

Furthermore, the characteristic tissue reactions that occur when large amounts of cortisone are given had developed. Lymphopenia, eosinopenia, atrophy of lymph nodes and the thymus gland, and marked neutrophilia were common. The adrenal glands were atrophic. The zona fasciculata of the adrenal gland was greatly reduced and the zona glomerulosa was considerably increased in size. Thus, amounts of the hormone which certainly proved toxic to the growing tumor, resulting in inhibition and even regression, also proved toxic to the organism itself.

Accordingly, the administration of cortisone was discontinued after the fifth day, which was the twelfth day after transfer of the tumor, for it was obvious that these test mice would no longer withstand the toxicity induced by the large amounts of the hormone being given. Within 2 days, however, after cessation of cortisone therapy, the persisting viable cells within the largely necrotic tumor began to grow, and as a result, growth of the tumor was resumed in all animals that survived.

Cortisone appeared to exert its influence directly upon the tumor cell, so modifying its metabolism as to result in its destruction. The characteristic large nuclei of the actively growing tumor with their wide parachromatin areas became, when subjected to cortisone, more compact and gradually pyknotic. The ratio of cytoplasmic area to nuclear substance gradually increased, the cell membrane ruptured, and the cell was destroyed.

We do not know why the several foci of viable cells persisted throughout the period of therapy. It is presumed that they either escaped the hormone or received it in such low concentrations as to make it ineffective. It may be presumed, too, that in low concentrations these surviving cells may have developed a tolerance to the hormone, permitting subsequent proliferation.

COMMENTS

In our experience, transplantation of a tumor, originally identified as a rhabdomyosarcoma, yielded to high blood levels of the adrenal steroid, cortisone. Growth of the tumor following implantation was completely inhibited by giving the hormone, and regression of well-established tumors was obtained. Tumor cells were largely destroyed. Two milligrams per

day, the equivalent of 100 mg. per kilogram of body weight, was more effective than smaller amounts. Tumor tissue, however, was not entirely destroyed, for in all animals, when cortisone therapy was discontinued, growth of the tumor, modified and retarded, was resumed.

Cortisone must be administered with caution, for if given for several days, the hormone may exert such physiologic changes in the animal organism as to cause death.

Cortisone is concerned with the metabolism of carbohydrates and proteins. It promotes gluconeogenesis from protein, it restricts the utilization of carbohydrates, and it depletes the body proteins. In large amounts it affects the electrolyte and water balance. Retention of sodium and water, together with increased excretion of potassium, takes place.

Then, too, cortisone, either through its influence upon animal metabolism or perhaps directly through its action upon connective tissue, inhibits or modifies the functions of the defense mechanisms of the body. As a result, repair of wounds is greatly restricted in the presence of high levels of cortisone.

Normal mice may die when subjected to prolonged increased concentration of cortisone in the blood. Mice bearing these transplanted muscle tumors and given a high intake of cortisone were anorexic, lost weight, and appeared physiologically depressed. Thus, it was apparent that there were optimal therapeutic amounts of cortisone that may safely be given. The immediate effects of the hormone upon the tumor, which we observed in 24 to 48 hours, were independent, we believe, of the physiologic depression exerted by the steroid upon the animal organism. We believe that these effects were due to some direct action upon the tumor cell, inciting its dissolution even as the lymphocyte is destroyed in the presence of this adrenal steroid.

SUMMARY

A study is reported of the influence exerted by large amounts of cortisone acetate upon the growth of an experimentally induced rhabdomyosarcoma. The tumor tested was in its sixty-eighth transplant generation and had lost the cytologic characteristics that had served to identify it as a muscle tumor.

Cortisone, when given in amounts equivalent to 100 mg. per kilogram of body weight per day, exerted marked inhibitory effects upon the tumor cell. Regression occurred and the size of the tumor was greatly reduced, although the tumor was never completely obliterated by the hormone. When cortisone therapy was withdrawn, viable foci of cells rapidly increased in size, and within a short time palpable tumors were restored.

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PLATES

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PLATE 81

FIGURE 1.—Section of the original tumor showing elongated anaplastic muscle fibers. Hematoxylin and eosin. × 250

FIGURE 2.—Section of the original tumor showing longitudinal striations and remnants of cross striations in the muscle cells. Hematoxylin and eosin. × 1,400

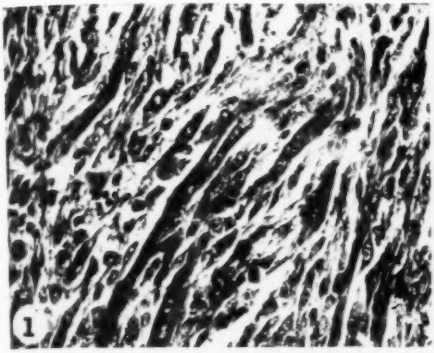
FIGURE 3.—Section of the tumor when made available for our study. Changes in the characteristics of the tumor during the successive transplantations since its origin are obvious. This pattern indicates reversion with loss of longitudinal and cross striations. Hematoxylin and eosin. × 325

FIGURE 4.—Sections of tumors of control animals (*A*, *B*, and *C*) and of those which had received cortisone (*D*, *E*, and *F*). *A*. Tumor seven days after transfer. *B*. Nine days after transfer. *C*. Eleven days after transfer. *D*. Nine days after transfer, an injection of cortisone having been given on the eighth and ninth days, respectively. *E*. Ten days after transfer, an injection of cortisone having been given on the eighth, ninth, and tenth days, respectively. *F*. Eleven days after transfer, an injection of cortisone having been given on the eighth, ninth, tenth, and eleventh days, respectively.

FIGURE 5.—Sections of a tumor nine days after transfer. The host was given 2 mg. of cortisone on the eighth and ninth days, respectively. Areas of necrosis are shown, and viable tumor cells surround muscle tissue of the host. Hematoxylin and eosin. × 325

FIGURE 6.—Section of the tumor shown in figure 4*E*. Degenerative changes are present in most cells. Nuclei are homogeneous and cell cytoplasm is indistinct. Hematoxylin and eosin. × 325

FIGURE 7.—Sections of the tumor shown in figure 4*F*. Further necrosis and general loosening of tumor tissue are evident. Much degeneration is present, but viable tumor cells still persist. Hematoxylin and eosin. × 325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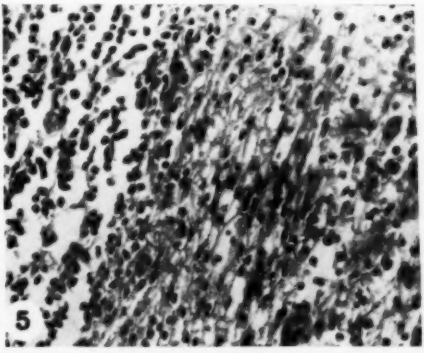
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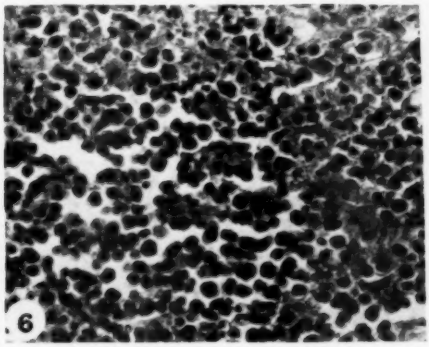
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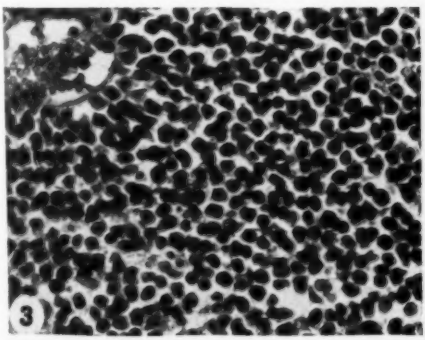
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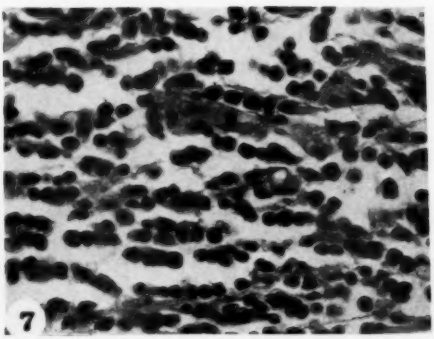
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STUDIES OF VARIOUS TESTS FOR MALIGNANT NEOPLASTIC DISEASES. VII. SERUM INHIBITORS OF CHYMOTRYPSIN AND TRYPSIN [1]

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INTRODUCTION

The ability of serum from healthy individuals to inhibit the proteolytic action of trypsin has been known for over 50 years [see Grob (*1*) and Cliffton (*2*) for a review of the earlier literature]. It has frequently been observed that high levels of this serum inhibitor may be found in persons with cancer, and it has accordingly been suggested that such a finding may be of use in the diagnosis of cancer. Studying 2,524 patients, Cliffton (*2*) found 68.1 percent elevated levels in cancer and believes that the level of inhibitor is useful in the diagnosis of cancer. Dillard and Chanutin (*3*) finding 76 percent elevated levels in 39 cancer cases do not attach any diagnostic significance to the values.

The antiproteolytic action of serum from healthy individuals as measured against chymotrypsin, appears to have been observed only recently by West (*4*). West (*4*), however, states that high values of this inhibitor were found in subjects having a variety of diseases and concluded that such a result was not of diagnostic value. Tauber (*5*), on the basis of a smaller sample, including 60 patients with cancer, found only a small incidence of abnormal values, but concluded that the test was of value in the diagnosis of advanced malignant disease.

It is evident that further study of levels of antiproteases in serum from healthy and diseased persons is desirable. We have studied 648 cases in which either inhibition of chymotrypsin, trypsin, or both, was measured and shall present data from the cases examined which suggest that neither inhibitor is regularly or exclusively elevated in cancer patients. Data on the properties and nature of the inhibitor(s) will be presented elsewhere.

[1] Received for publication October 16, 1951.

EXPERIMENTAL PROCEDURE

Selection of Cases

The method of selecting cases has been reported in detail in a previous paper in this series (*6*). The normal group in this study was comprised of individuals between the ages of 19 and 65. All of these people appeared to be in good health, and each had a normal temperature, blood count (complete), hemoglobin concentration and chest X-ray. The group of individuals with miscellaneous diseases was chosen to include some individuals with diseases that might be difficult to distinguish clinically from neoplastic disease, and to include a variety of other acute and chronic diseases. The records of all these cases were carefully reviewed to determine how the clinical diagnoses were established. The individuals with benign neoplasms and those with malignant neoplasms were selected to give wide histogenetic representation. In the latter group approximately half had known metastases. All cases of neoplastic disease were proved by microscopic examination of the tissues removed at biopsy, operation, or autopsy. All specimens of blood were taken prior to operation, irradiation, or specific chemical therapy. No case is reported wherein there was a blood transfusion within 1 week before testing. In all groups, specimens of blood were taken after an overnight fast and were tested on the same day.

The chymotrypsin series was composed of 112 normal individuals, 55 with benign neoplasms, 153 with miscellaneous nonneoplastic diseases, and 136 with malignant neoplasms, of whom 60 had proved metastases. The trypsin series was composed of 66 normal individuals, 53 with benign neoplasms, 132 with miscellaneous nonneoplastic diseases and 115 with malignant neoplasms, of whom 51 had proved metastases.

Determination of Inhibitor

We have used the method of Kunitz (*7*), devised for measuring soybean trypsin inhibitor, for determination of both chymotrypsin and trypsin inhibitor. In the case of chymotrypsin, 1 ml. of diluted serum (1:100 dilution in $\frac{M}{10}$ phosphate buffer, pH 7.60) and 1 ml. of casein solution (1 percent) are equilibrated at 37° C. At zero time 1 ml. of a solution of crystalline enzyme [2] (approximately 0.025 mg. of protein in $\frac{M}{400}$ HCl) is added and proteolysis ensues. After 20 minutes at 37° C., 3 ml. of 5 percent trichloroacetic acid is added, which stops the reaction and precipitates the undigested casein. The tubes are allowed to stand 1 hour, are centrifuged, and the concentration of split products in the supernatant is measured in the Beckman DU spectrophotometer by the density at 280 mμ. Reference to a standard curve prepared at the same time by using 0.2, 0.4, 0.6, 0.8, and 1.0 ml. of enzyme solution permits direct calculation of the amount of chymotrypsin inhibited. The results are expressed as micrograms of enzyme inhibited by 0.01 ml. of serum.

[2] Worthington Biochemical Co.

For trypsin inhibitor, the method is varied slightly. One ml. of serum diluted as above is added to 1.0 ml. of trypsin [3] solution (ca. 0.035 mg. per ml. in $\frac{M}{400}$ HCl). After 10 minutes at 37° C., 1.0 ml. of casein solution is added. The remainder of the assay is carried out as for chymotrypsin inhibitor.

The concentration of chymotrypsin and trypsin in the enzyme solutions is measured by the density at 280 mμ using the factors 0.500 and 0.585 as supplied by Kunitz (7).

The reproductibility of this method has been studied in two 25-replicate experiments, in which the coefficients of variation were found to be 0.011 for chymotrypsin inhibitor and 0.012 for trypsin inhibitor. The effect of storing serum in the refrigerator for 3 days prior to testing has been investigated. Statistical analysis showed no significant differences. Only fresh sera, however, were used in this series.

RESULTS

The results obtained in this study are shown in summary in table 1.

TABLE 1.—*Summary of test results, elevation of antiprotease levels in serum*

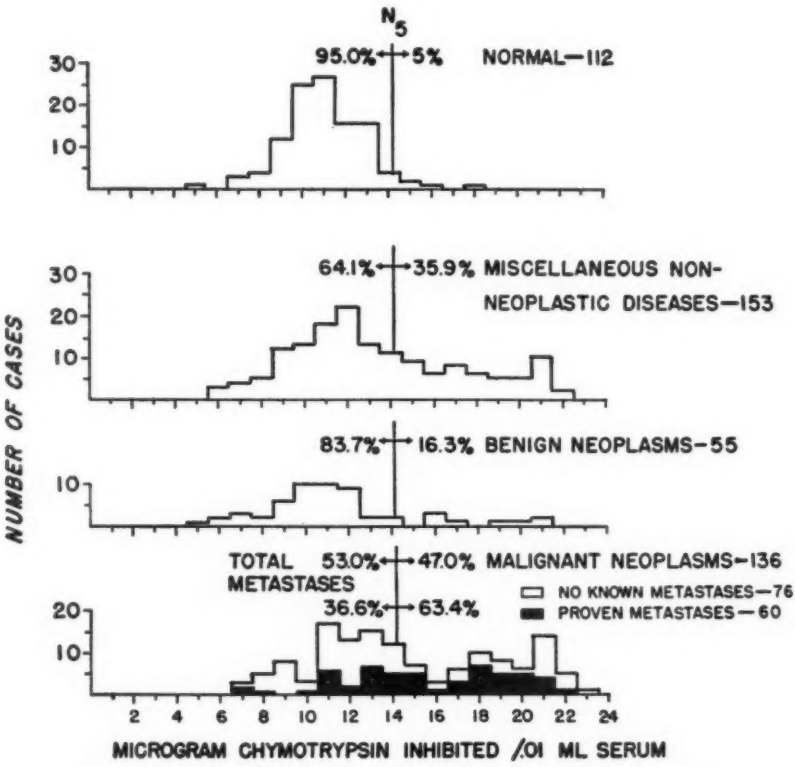
Classification	Antichymotrypsin		Antitrypsin	
	Number of cases	Percent elevated	Number of cases	Percent elevated
Normal	112	[1] 5.0	66	[1] 5.0
Miscellaneous	153	35.9	132	43.1
Benign neoplasms	55	16.3	53	17.0
Malignant neoplasms	136	47.0	115	60.0

[1] By definition: See text.

Text-figures 1 and 2 show the distribution of test results among the major classifications studied (normal, malignant neoplasms, benign neoplasms, and miscellaneous diseases). The point N_5 of the normal group is computed as described in paper I of this series [Eriksen, Ellerbrook, and Lippincott (6)] and represents a dividing point permitting an arbitrary level of 5 percent false-positive results. If a test result is above the N_5 point (14.2 [4] for chymotrypsin inhibitor, 17.6 [4] for trypsin) it is considered "positive." According to this point of division in the chymotrypsin series, 53 percent of those patients with malignant neoplasms failed to give a "positive" test, while 35.9 percent of those with miscellaneous diseases and 16.3 percent of those with benign neoplasms gave a false-positive result. In the trypsin series the percentages are 40.0, 43.1, and 17.0, respectively. Complete data are shown in appendix tables 4, 5, and 6.

In tables 2 and 3 a subdivision of the results by organ system is shown. These tables, as well as the text-figures, show that a much higher incidence of abnormal values was found among those cancer patients with metastases than is the case when metastases were not detected.

[3] Crystallized trypsin, Armour.
[4] Confidence interval of N_5: chymotrypsin series, ±0.6; trypsin series, ±1.0.

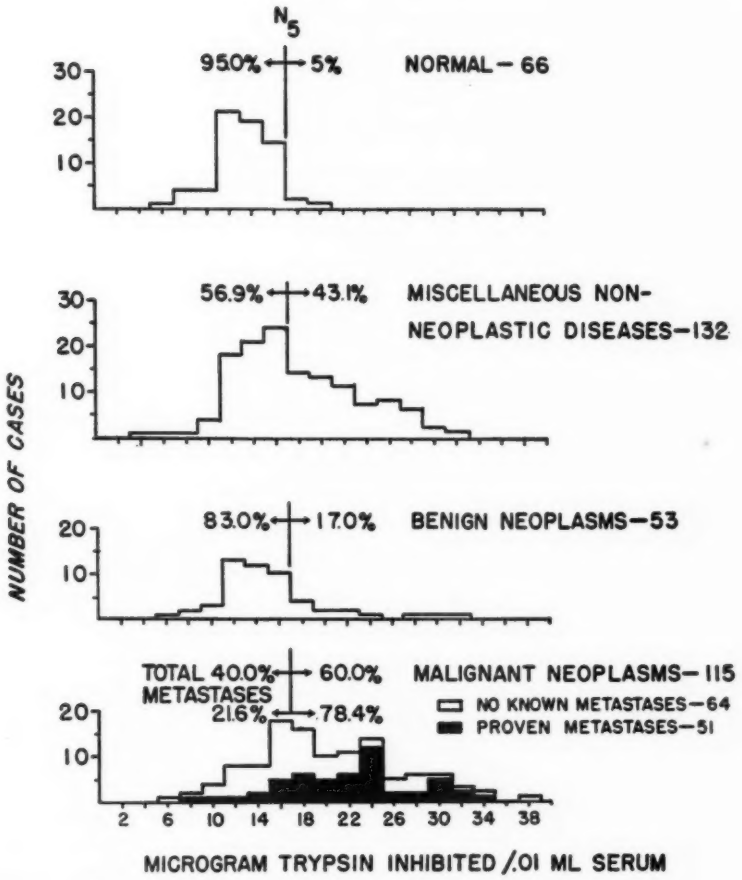


TEXT-FIGURE 1.—Distribution of test values, chymotrypsin inhibitor.

DISCUSSION

It appears from these data that the existence of a malignant neoplasm is not a specific variable in the elevation of the inhibitors in sera. The lack of specificity of the reaction is further demonstrated if results on cancer patients are compared with results from patients having nonneoplastic diseases in the same organ system (tables 2 and 3). Such comparison shows essentially similar results, except for kidney, ureter, bladder, and prostate gland, where the presence of a malignant neoplasm is accompanied by a somewhat higher incidence of abnormal values. The higher incidence of positive results among those patients known to have metastases tends to confirm the results of Tauber (*5*), but is in contrast to the findings of Cliffton (*2*). Cases where the lesions involve the kidney, ureter, bladder, or prostate gland constitute exceptions, the incidence of positive results being uniformly high.

In addition to lack of specificity, the sensitivity of the reaction is very low. A test suitable for mass screening purposes has been defined as one that gives a "positive" result on at least 90 percent of cancer patients with



TEXT-FIGURE 2.—Distribution of test values, trypsin inhibitor.

5 percent "false positive" among an apparently well population [Dunn and Greenhouse (*8*)]. The level of serum inhibitor(s) of trypsin and chymotrypsin fails to meet this criterion.

The distributions of test results for chymotrypsin and trypsin inhibitors very closely parallel each other in sera from individuals known to have a disease process. In addition to the general distribution of results, in individual cases the level of inhibitors is clearly related. In 166 of 186 cases the level of both inhibitors was either above or below its respective value for N_5 ($\pm$ confidence interval). These observations are consistent with the hypothesis that only a single substance, a proteolytic inhibitor, is involved rather than two distinct entities; *i. e.*, chymotrypsin and trypsin inhibitors. Further studies on this question are in progress and will be described in a subsequent publication.

TABLE 2.—*Distribution of test results by organ system—chymotrypsin inhibitor*

Organ system	Malignant neoplasms			Miscellaneous		
	Number of cases	Number abnormal	Percent abnormal	Number of cases	Number abnormal	Percent abnormal
Alimentary:						
W [1]	22	17	77. 2			
W/O [2]	25	9	36. 0			
Total	49	26	51. 0	34	23	67. 6
Respiratory:						
W	10	8	80. 0			
W/O	5	1	20. 0			
Total	15	9	60. 0	7	4	57. 0
Skin and breast:						
W	10	2	20. 0			
W/O	19	2	10. 5			
Total	29	4	13. 8	21	1	4. 8
Cervix, uterus, ovary, testis, placenta:						
W	3	2	66. 7			
W/O	12	3	25. 0			
Total	15	5	33. 3	30	8	26. 6
Kidney, ureter, bladder, prostate gland:						
W	9	7	77. 8			
W/O	12	9	75. 0			
Total	21	16	76. 2	21	12	38. 8
Other:						
W	6	2	33. 3			
W/O	3	0	0			
Total	9	2	22. 2	30	7	23. 3
Total:						
W	60	38	63. 4			
W/O	76	26	34. 2			
Total	136	64	47. 0	153	55	35. 9

[1] With known metastases.
[2] Without known metastases.

TABLE 3.—*Distribution of test results by organ system—trypsin inhibitor*

Organ system	Malignant neoplasms			Miscellaneous		
	Number of cases	Number abnormal	Percent abnormal	Number of cases	Number abnormal	Percent abnormal
Alimentary:						
W [1]	22	20	91. 0			
W/O [2]	17	9	52. 9			
Total	39	29	74. 4	30	24	80. 0
Respiratory:						
W	10	9	90. 0			
W/O	3	2	66. 7			
Total	13	11	84. 6	5	3	60. 0
Skin and breast:						
W	7	1	14. 3			
W/O	17	4	23. 5			
Total	24	5	20. 8	19	3	15. 8
Cervix, uterus, ovary, testis, placenta:						
W	2	2	100. 0			
W/O	14	8	57. 1			
Total	16	10	62. 4	25	6	24. 0
Kidney, ureter, bladder, prostate gland:						
W	6	5	83. 3			
W/O	11	7	63. 6			
Total	17	12	70. 6	27	12	44. 4
Other:						
W	4	2	50. 0			
W/O	2	0	0			
Total	6	2	33. 3	26	9	34. 6
Total:						
W	51	40	78. 4			
W/O	64	29	45. 4			
Total	115	69	60. 0	132	57	43. 1

[1] With known metastases.
[2] Without known metastases.

SUMMARY

Chymotrypsin inhibitor levels and trypsin inhibitor levels have been determined in 456 cases and 365 cases, respectively. A normal control group was included in each. Gastrointestinal, urinary, and respiratory disorders account for the bulk of the abnormal values obtained. Positive test values were found as follows: chymotrypsin series—malignant neoplasms 47.0 percent, benign neoplasms 16.3 percent, miscellaneous nonneoplastic diseases 35.9 percent; trypsin series—60.0, 17.0, and 43.1 percent, respectively.

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APPENDIX

TABLE 4.—*Patients with malignant neoplastic diseases*

ALIMENTARY

Specimen No.	Diagnosis	Age	Sex	Known metastases		Xt[1]	T[1]
				Yes	No		
5066	Carcinoma esophagus	50	M		x	21.4	
5351	Carcinoma stomach	76	M	x		15.2	
5488	do	79	M	x		21.4	28.3
5528	do	78	M	x		20.8	27.0
5561	do	43	F	x		17.6	23.2
5594	do	73	F	x		15.0	22.1
5836	do	64	M	x		17.6	23.4
5861	do	41	F	x		14.7	19.2
5915	do	62	M	x		21.8	31.9
6134	do	60	M	x		12.5	18.8
6149	do	70	M	x		13.8	21.5
5561	do	43	F	x		17.6	23.2
6463	do	69	M	x			21.3
6195	do	61	F	x		17.5	23.4
5308	Carcinoma colon	61	F		x	12.2	
5346	do	39	F	x		11.9	18.5
5630	do	67	M	x		21.2	29.3
5641	Carcinoma sigmoid	76	F	x		18.3	24.7
6435	do	72	F	x			29.6
5112	Carcinoma cecum	41	M	x		18.2	
6208	do	72	F		x	15.7	22.5
5035	Carcinoma rectum	81	F		x	12.5	
5197	do	67	M		x	18.4	
5068	do	76	M	x		19.9	
5481	do	70	F		x	17.2	
5366	do	70	M	x		12.7	22.2
5382	do	72	M		x	10.6	13.7

See footnotes at end of table.

TABLE 4.—*Patients with malignant neoplastic diseases*—Continued

ALIMENTARY—Continued

Specimen No.	Diagnosis	Age	Sex	Known metastases		Xt[1]	T[2]
				Yes	No		
5504	Carcinoma rectum	79	M	-------	x	12.7	18.1
5517	------do	59	M	x	-------	19.5	30.1
5565	------do	78	F	-------	x	14.1	19.3
5592	------do	60	M	-------	x	13.2	19.4
5191	------do	65	F	-------	x	9.2	------
5818	------do	57	M	-------	x	15.1	22.1
5820	------do	66	M	-------	x	12.7	17.6
5831	------do	83	M	x	-------	16.9	19.1
5840	------do	67	F	-------	x	10.5	13.7
5885	------do	70	F	-------	x	12.4	18.1
5894	------do	81	M	-------	x	19.8	15.1
5903	------do	67	M	-------	x	11.7	15.9
5935	------do	59	M	-------	x	14.3	19.1
6229	------do	58	M	-------	x	9.0	14.2
6266	------do	61	M	-------	x	18.6	------
6276	------do	60	M	x	-------	------	19.7
6428	------do	55	F	x	-------	------	15.1
5951	Carcinoma bile duct	74	M	-------	x	20.6	27.7
5200	Carcinoma pancreas	61	M	x	-------	20.5	------
5742	------do	68	M	-------	x	12.5	15.6
5919	------do	70	M	-------	x	17.1	22.7
2966	Squamous-cell carcinoma, lip and tongue.	66	M	-------	x	11.4	------
5957	------do	61	M	x	-------	13.3	17.4
5127	Squamous cell carcinoma, tongue.	61	M	-------	x	11.2	------
6297	Squamous-cell carcinoma, lip	59	M	-------	x	------	15.2

RESPIRATORY

5457	Bronchiogenic carcinoma	51	M	x	-------	18.9	24.9
5409	------do	53	F	x	-------	11.0	16.3
5503	------do	48	M	x	-------	20.0	33.1
5566	------do	49	M	x	-------	13.9	19.0
5117	------do	55	M	-------	x	11.3	------
5017	------do	62	M	x	-------	19.3	------
5052	------do	71	M	x	-------	22.1	------
5019	------do	44	M	x	-------	19.3	------
5838	------do	58	M	-------	x	8.3	12.5
5839	------do	61	M	x	-------	14.3	17.9
5845	------do	45	M	x	-------	14.6	20.2
5865	------do	45	M	-------	x	21.0	28.6
6425	------do	51	M	x	-------	------	24.3
6423	------do	34	M	x	-------	------	23.5
6371	------do	63	M	x	-------	------	26.6
6182	------do	60	M	x	-------	18.5	24.6
5130	Squamous-cell carcinoma, larynx.	59	F	-------	x	10.8	------
6104	------do	53	M	-------	x	13.6	21.4

See footnotes at end of table.

TABLE 4.—*Patients with malignant neoplastic diseases*—Continued

KIDNEY, URETER, BLADDER, AND PROSTATE GLAND

Specimen No.	Diagnosis	Age	Sex	Known metastases		Xt[1]	T[2]
				Yes	No		
5257	Carcinoma prostate gland	76	M	x		17.3	
5118	do	66	M		x	21.4	
5015	do	80	M		x	14.6	
5012	do	74	M	x		12.2	
5316	do	62	M		x	17.3	
5318	do	59	M	x		17.7	
5518	do	70	M		x	11.1	14.3
5608	do	77	M	x		17.0	24.5
5642	do	74	M		x	17.8	24.9
5717	do	67	M		x	21.2	25.9
6240	do	78	M	x		22.3	30.8
6302	do	85	M		x		27.4
6390	do	71	M		x		16.3
5980	Carcinoma kidney	55	M		x	20.5	31.3
5629	do	66	M	x		14.9	21.8
6114	do	65	M		x	19.2	25.0
5309	Carcinoma bladder	71	M		x	12.9	
5577	do	74	M		x	20.7	27.2
5837	do	71	M		x	7.9	10.4
5956	do	44	M	x		10.6	11.7
6036	do	81	M	x		20.0	23.6
6038	do	63	M	x		21.9	31.0
6191	do	45	M		x	22.0	29.0
6363	do	57	M		x		7.6

CERVIX, UTERUS, TESTIS, OVARY, AND PLACENTA

5410	Carcinoma cervix	53	F		x	13.5	24.4
5430	do	80	F		x	18.3	33.6
5020	do	54	F	x		18.6	
5121	do	44	F	x		15.6	
5774	do	69	F		x	11.8	18.6
5775	do	76	F		x	19.3	25.5
5799	do	54	F		x	12.5	18.2
5866	do	45	F		x	8.8	15.1
6436	do	47	F		x		17.7
6400	do	39	F	x			25.1
6358	do	44	F		x		6.9
5448	Carcinoma uterus	59	F		x	10.8	14.1
5359	do	71	F		x	12.3	21.0
5406	do	53	F		x	8.6	15.0
5538	do	60	F		x	13.5	17.1
5893	do	47	F		x	15.7	20.6
6136	do	72	F	x		13.3	22.4
6046	Malignant teratoma, testis	15	M		x	10.4	14.3

See footnotes at end of table.

TABLE 4.—*Patients with malignant neoplastic diseases*—Continued

SKIN AND BREAST

Specimen No.	Diagnosis	Age	Sex	Known metastases		Xt[1]	T[2]
				Yes	No		
5102	Carcinoma, breast	59	F	x		6.7	
5212	do	54	F	x		11.3	
5144	do	69	F		x	12.2	
5192	do	61	F	x		12.5	
5358	do	70	F		x	20.8	38.8
5357	do	76	F		x	13.8	19.5
2967	do	78	F		x	12.1	
5513	do	44	F	x		8.2	10.6
5516	do	68	F	x		11.1	14.3
5670	do	62	F		x	8.9	11.7
5644	do	63	F		x	8.6	10.7
6037	do	64	F		x	8.9	12.1
6079	do	43	F	x		7.3	8.9
6117	do	32	F		x	9.8	14.7
6189	do	55	F	x		11.2	16.6
6255	do	50	F		x	7.5	12.6
6478	do	54	F		x		11.3
6475	do	44	F		x		11.8
6467	do	57	F	x			15.5
5099	Sarcoma, breast	78	F	x		23.1	
5101	Basal-cell carcinoma, rectum	64	M		x	12.0	
5848	Basal-cell carcinoma, scalp	48	F		x	8.4	15.2
6127	Basal-cell carcinoma, nose	40	M		x	20.5	12.5
6093	Basal-cell carcinoma, ear	67	F		x	7.4	10.9
6169	Basal-cell carcinoma, face	64	M		x	10.5	16.6
6183	do	51	M		x	12.7	18.8
5427	Squamous-cell carcinoma, face	77	F		x	11.2	18.2
5545	Squamous-cell carcinoma, penis	78	M		x	12.1	16.9
5077	Squamous-cell carcinoma, skin	92	M		x	13.9	
6005	Squamous-cell carcinoma, nose	78	M	x		14.3	17.5
6184	Squamous-cell carcinoma, vulva	68	F		x	8.5	15.2
5682	Melanoma, malignant	70	F	x		13.5	18.8

OTHER

5872	Ewing's sarcoma	25	F		x	10.9	15.9
5592	Lymphosarcoma	82	F	x		21.2	29.2
5937	do	73	M	x		11.0	15.7
5645	do	57	M	x		10.1	14.7
5720	do	65	M	x		19.5	23.0
5924	Chronic myelogenous leukemia	60	M		x	11.8	17.2
5011	Hodgkin's disease	73	F	x		12.6	
5062	Carcinoma, parotid gland	22	F		x	12.0	
5096	Carcinoma, submaxillary gland	80	M	x		13.4	

[1] Xt=gamma chymotrypsin inhibited/0.01 ml. serum.
[2] T=gamma trypsin inhibited/0.01 ml. serum.

TABLE 5.—*Patients with benign neoplastic diseases*

Specimen No.	Diagnosis	Age	Sex	Xt[1]	T[1]
5867	Leiomyoma, esophagus	28	F	9.5	16.6
6271	Mixed tumor, submaxillary gland	60	F	------	13.1
6041	Chondroma, trachea	72	M	12.4	20.1
5407	Papilloma, larynx	36	M	10.1	15.1
6075	------do	51	M	10.5	14.6
5098	Leiomyoma, uterus	47	F	10.8	------
5093	------do	32	F	13.0	------
5104	------do	59	F	12.2	------
5259	------do	42	F	10.7	------
5196	------do	39	F	10.6	------
5348	------do	54	F	11.7	------
5360	------do	46	F	20.3	31.9
5113	------do	55	F	14.3	------
5310	------do	40	F	11.6	------
5539	------do	40	F	8.7	12.7
5574	------do	39	F	9.9	17.9
5575	------do	65	F	21.2	29.2
5872	------do	54	F	8.3	14.0
5849	------do	47	F	6.1	8.4
6012	------do	40	F	10.0	12.9
6025	------do	47	F	10.4	16.2
6043	------do	26	F	21.1	28.4
6254	------do	43	F	9.0	11.2
6270	------do	50	F	------	10.3
6294	------do	44	F	------	14.4
6232	------do	35	F	10.1	17.0
6206	------do	39	F	10.5	17.0
6186	------do	47	F	12.2	13.0
6185	------do	48	F	7.4	11.7
6432	------do	30	F	------	14.5
6431	------do	48	F	------	9.3
6375	------do	45	F	------	12.8
6359	------do	45	F	------	16.5
5983	Fibroma, ovary	39	F	8.2	12.4
5022	Fibroadenoma, breast	28	F	13.1	------
5210	------do	28	F	10.5	------
5527	------do	24	F	11.9	16.5
5748	------do	26	F	7.4	11.1
6091	------do	61	F	10.5	12.6
6352	------do	23	F	------	11.7
5229	Cystadenoma, breast	28	F	19.1	------
5007	Intraductal papilloma, breast	21	F	10.9	------
6410	------do	40	F	------	12.2
5209	Lipoma	49	F	10.2	------
5500	------do	40	F	4.6	8.8
5519	------do	65	M	16.1	21.5
5563	------do	42	F	9.4	15.2
5923	------do	39	M	9.0	13.1
6044	------do	49	F	9.7	12.9
6076	------do	57	M	9.8	12.9
6252	------do	62	M	7.3	12.9
2965	Meningioma	50	M	15.6	------
5433	------do	62	F	16.9	10.9
5846	Papilloma, choroid plexus	63	M	13.9	19.5
6008	Angioma, brain	22	M	15.9	21.1
6272	Angioma, pia-arachnoid	50	M	------	24.2
5921	Osteoma	36	M	6.0	5.5
6027	Osteoid osteoma	63	M	10.8	14.7
6101	Chondrosteoma	44	F	9.5	16.1
6251	Osteochondroma, ilium	37	F	9.0	14.7
6464	Neurofibroma, sacrum	54	M	------	15.2

See footnotes at end of table.

TABLE 5.—*Patients with benign neoplastic diseases*—Continued

Specimen No.	Diagnosis	Age	Sex	Xt[1]	T[2]
5643	Adenoma, thyroid gland	55	M	11.6	15.5
5241	do	26	F	12.1	------
5080	do	44	F	11.3	------
6336	Papillary adenoma, thyroid gland	26	F	------	16.8
6298	do	35	F	------	13.7
6479	Rhabdomyoma	37	M	------	14.3
6205	Verruca senilis	63	F	12.1	17.3
6209	do	58	F	9.0	14.5

[1] Xt—gamma chymotrypsin inhibited/0.01 ml. serum.
[2] T—gamma trypsin inhibited/0.01 ml. serum.

TABLE 6.—*Patients with miscellaneous nonneoplastic diseases*

ALIMENTARY

Specimen No.	Diagnosis	Age	Sex	Xt[1]	T[2]
5064	Gastric ulcer	64	M	22.8	------
5501	do	68	M	16.4	22.9
5610	do	80	M	12.9	20.7
5933	do	86	M	17.9	23.7
6128	do	70	M	11.5	17.8
6011	do	70	M	20.4	27.8
6047	do	43	F	8.5	11.4
6092	do	49	M	12.0	17.4
6387	do	38	F	------	26.6
6230	do	52	M	16.7	24.7
5228	Duodenal ulcer	62	M	20.6	------
5480	do	63	M	8.0	------
5963	do	36	M	8.5	11.9
5722	do	59	M	17.0	20.6
6130	do	60	M	12.6	20.2
6234	do	59	M	12.7	20.4
5060	do	58	M	15.3	------
5067	Acute cholecystitis	55	F	17.5	------
5013	do	79	M	15.9	------
6006	do	31	F	20.6	27.0
5485	Chronic cholecystitis	75	F	19.0	25.2
5640	do	80	F	20.6	27.1
6129	do	56	M	18.0	15.9
6002	do	78	F	20.0	26.4
5075	Infectious hepatitis	67	M	14.7	------
6282	do	56	M	------	22.2
5570	Liver abscess	38	M	18.6	25.6
5595	do	56	M	21.4	28.8
5932	Diverticulum, sigmoid	88	M	15.5	21.5
5365	Periduodenal adhesions	62	M	18.3	27.0
5079	Amebic colitis	57	F	20.6	------
5896	Ulcerative colitis	31	M	19.7	26.4
6003	Regional ileitis	46	F	10.7	14.1
5039	Steatorrhea	62	M	10.7	------
5982	Leukoplakia, lip	62	M	8.5	12.6
5851	do	60	M	15.0	19.0
6295	Chronic inflammation, salivary gland	23	F	------	22.8
6420	Prolapse, gastric mucosa	73	F	------	19.6
6333	Atrophic gastritis	72	F	------	29.2

See footnotes at end of table.

TABLE 6.—*Patients with miscellaneous nonneoplastic diseases*—Continued

RESPIRATORY

Specimen No.	Diagnosis	Age	Sex	Xt [1]	T [2]
5063	Bronchiectasis	21	F	17.5	------
5029	Bronchopneumonia	77	M	14.6	------
6179	Lobar pneumonia	85	M	17.0	23.1
5499	Pneumonitis	43	M	16.8	22.8
6180	Infarct, lung	60	M	13.7	21.0
6124	Influenza	42	F	9.7	13.7
5438	Severe cold	43	M	10.5	13.9

KIDNEY, URETER, BLADDER, AND PROSTATE GLAND

6024	Chronic pyelonephritis	62	M	12.9	17.5
5116	----do	25	M	21.0	------
6382	----do	50	F	------	11.6
5432	Acute pyelonephritis	61	M	16.2	22.7
5061	Pyelonephrosis	48	F	7.2	------
5171	Calculus, kidney	39	M	21.3	------
5195	Calculus, ureter	56	M	10.3	------
5739	----do	73	M	19.0	24.2
5199	Calculus, bladder	70	M	21.3	------
5041	----do	66	M	17.0	------
5097	Hypertrophy, prostate gland	67	M	12.5	------
5145	----do	67	M	11.3	------
5321	----do	68	M	11.9	------
5317	----do	85	M	15.0	------
6148	----do	73	M	11.3	16.4
5350	----do	60	M	15.0	------
5435	----do	70	M	8.3	13.9
5934	----do	80	M	12.1	15.7
5917	----do	76	M	14.5	18.9
5719	----do	70	M	19.1	21.5
6173	----do	66	M	8.1	12.6
6204	----do	65	M	12.6	19.1
6215	----do	68	M	14.0	18.1
5672	----do	80	M	10.9	14.9
6256	----do	82	M	11.1	14.8
6376	----do	75	M	------	16.9
6383	----do	71	M	------	21.1
6388	----do	83	M	------	23.0
6460	----do	73	M	------	17.7
6465	----do	65	M	------	17.2
6474	----do	73	M	------	14.3
5891	Hypertrophy prostate gland, and pyelonephritis	59	M	20.7	27.4
5665	Chronic prostatitis	60	M	6.0	10.8
5380	Cystitis	62	M	9.1	7.5
5953	Chronic cystitis	56	M	21.7	31.3
5415	Cystocele and rectocele	53	F	13.6	19.8
5942	----do	55	F	10.2	15.5

See footnotes at end of table.

TABLE 6.—*Patients with miscellaneous nonneoplastic diseases*—Continued

CERVIX, UTERUS, OVARY, TESTIS, AND PLACENTA

Specimen No.	Diagnosis	Age	Sex	Xt[1]	T[1]
5514	Chronic salpingitis	25	F	12.0	17.2
2949	Chronic endometritis	48	F	9.7	------
5567	------do	36	F	10.5	16.4
5008	Chronic cervicitis	19	F	15.6	------
5486	------do	51	F	10.5	16.8
5515	------do	37	F	9.8	13.6
5692	------do	63	F	15.3	20.6
5779	------do	73	F	19.3	25.2
6022	------do	66	F	10.2	12.5
6080	------do	28	F	9.6	14.3
6350	------do	34	F	------	6.7
6374	------do	73	F	------	16.3
6414	------do	26	F	------	10.4
6445	------do	55	F	------	12.9
6397	Chronic cystic cervicitis	58	F	------	11.7
5036	Chronic cervicitis and prolapse uterus	42	F	10.5	------
5156	Endometrial hyperplasia	34	F	12.4	------
6010	------do	24	F	6.8	10.4
6021	------do	46	F	9.6	13.0
6074	------do	26	F	15.1	20.8
6115	------do	23	F	9.0	12.8
5055	------do	30	F	11.5	------
5170	------do	40	F	12.0	------
5072	------do	42	F	16.1	------
5651	------do	56	F	11.9	16.9
5054	Rectovaginal fistula	40	F	12.0	------
5264	Endometrium, atrophy	72	F	13.8	------
5172	Adenomyosis, uterus	40	F	11.4	------
5094	------do	44	F	13.0	------
5873	------do	51	F	5.8	13.6
6253	Retention of secundines	32	F	18.2	25.0
5038	------do	36	F	9.9	------
5714	Chronic epididymitis	39	M	11.5	14.0
6248	Pregnant uterus	47	F	------	20.4
6265	Cervical polyp	47	F	10.7	------
6320	Endometriosis, ovary	22	F	------	17.6
6443	------do	37	F	------	16.4
6353	Functional bleeding	32	F	------	16.5
6381	Menorrhagia and prolapse	46	F	------	13.3
5211	Tubo-ovarian abscess	30	F	20.5	------
5119	------do	16	F	21.2	------

SKIN AND BREAST

5576	Abscess, breast	21	F	16.6	23.0
5174	Cystic disease, breast	28	F	11.8	------
2948	------do	43	F	12.1	------
5198	------do	46	F	13.8	------
5056	------do	53	F	12.0	------
5399	------do	44	F	9.0	18.1
5143	------do	48	F	12.9	------
5498	------do	38	F	10.5	17.1
5581	------do	39	F	9.4	16.4
5941	------do	48	F	8.5	13.6
5931	------do	47	F	9.7	11.1
5871	------do	33	F	6.7	13.6
5802	------do	43	F	11.6	18.2
6322	------do	26	F	------	12.5
6422	------do	22	F	------	15.5

See footnotes at end of table.

TABLE 6.—*Patients with miscellaneous nonneoplastic diseases*—Continued

SKIN AND BREAST

Specimen No.	Diagnosis	Age	Sex	Xt [1]	T [2]
6433	Cystic disease, breast	45	F	------	11.2
5819	------do	30	F	8.2	11.1
5666	------do	43	F	9.4	12.7
5598	------do	28	F	10.6	16.6
6233	------do	37	F	6.4	12.7
6125	Gynecomastia	15	M	9.4	15.4
5449	Intraductal hyperplasia	42	F	11.3	14.1
5681	------do	73	F	11.5	14.2
6264	Epidermoid cyst	72	F	10.0	------
6421	Calcified epidermoid cyst	8	M	------	10.4

OTHER

6354	Exostosis, os calcis	66	M	------	12.0
5482	Fracture	64	M	17.3	------
6214	Exostosis	41	M	8.9	13.7
6281	Syphilitic osteitis, rib	42	F	------	18.9
5489	Pernicious anemia	81	F	14.3	------
6360	Felty's syndrome	62	M	------	20.4
5173	Hyperthyroidism	60	F	13.8	------
6026	Hyperplasia, thyroid gland	61	F	9.7	11.4
6170	Thyrotoxicosis	34	F	12.1	20.0
6384	Diffuse hyperplasia, thyroid gland	23	F	------	14.7
6426	Colloid goiter	55	M	------	13.9
5177	Diabetes mellitus	55	F	10.6	------
5901	Thyroglossal duct, cyst	60	M	12.2	16.3
5591	Dacryocystitis	59	F	8.4	15.2
5016	Diaphragmatic hernia	72	F	19.7	------
5364	Myositis ossificans	17	M	11.6	22.2
6116	------do	32	M	10.1	15.2
5378	Retroperitoneal tuberculosis	47	M	14.1	16.2
5081	Synovial cyst, foot	33	M	11.8	------
5122	Hyperplasia, lymph node	33	M	13.3	------
5473	Lymphadenitis	28	M	13.5	11.4
5260	Suppurative lymphadenitis	41	F	12.8	------
5083	Chronic tonsillitis	33	F	12.2	------
2997	Meningeal adhesions	27	M	12.7	------
5590	Cerebral infarction	59	M	13.7	21.6
6368	Intracranial hemorrhage	47	M	------	18.2
5194	Hypertensive cardiovascular disease	46	M	8.5	------
5272	------do	47	M	14.0	------
5452	------do	54	F	6.9	3.6
5043	Arteriosclerotic heart disease	77	M	12.1	------
5458	Coronary thrombosis	57	F	13.4	16.6
5801	------do	50	M	11.4	15.7
5863	Aneurysm, aorta	61	M	19.9	26.4
5854	Gangrene, foot	59	F	12.6	16.8
5546	------do	84	F	11.2	16.3
6237	Coronary insufficiency	75	M	17.3	23.4
6299	Myocardial decompensation, origin undetermined	61	M	------	15.9
6398	Hemiplegia	80	M	------	29.5
6372	------do	56	M	------	13.1

[1] Xt=gamma chymotrypsin inhibited /0.01 ml. serum.
[2] T=gamma trypsin inhibited /0.01 ml. serum.

OVARIAN TUMORIGENESIS FOLLOWING INTRASPLENIC TRANSPLANTATION OF OVARIES FROM WEANLING, YOUNG ADULT, AND SENILE MICE [1] [2]

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Ovarian tumors have been induced frequently in castrated rats (*1*, *2*, *3*) and mice (*4–8*, *9*) but not in guinea pigs (*10*, *11*) following intrasplenic or intrapancreatic transplantation of ovarian tissue. In these sites, estrogen secreted by the ovary passes through the portal system into the liver, where it is inacti vated (*12*). With a fall in systemic estrogen, the anterior lobe of the pituitary gland secretes increased amounts of gonadotrophic hormone (*13*, *14*, *15*). It has been said that a continuation of this hormonal imbalance is responsible for the induction of ovarian tumors among castrated rats and mice that have received intrasplenic grafts of ovarian tissue (*3*, *6*, *16*). It is well known that in women as ovarian function declines in old age, there is a fall in the level of estrogen and a general rise in the level of circulating gonadotrophin. In the presence of this hormonal imbalance, an increased incidence of ovarian tumors would be expected. Since this is not known to occur, Li and Gardner (*8*) have suggested that either "the ovaries of old animals may be refractory to stimulation by even augmented amounts of gonadotrophin or the level of gonadotrophin may be inadequate in quality or quantity."

In experiments where age of ovary and of host was varied, about the same tumor incidence was observed whether old or young ovaries were transplanted intrasplenically to young castrated mice (*8*). In the latter investigations, the old ovaries varied in age from 340 to 409 days, although in one instance a 491-day ovary was transplanted. In a study of ovarian aging processes in mice of different strains, Loeb (*17*) divided the life of the mouse into four periods: "period 1—from birth up to 9 months of age; period 2—from 10 to 14 months; period 3—from 15 to 19 months; and period 4—20 months and older." Since most of the old ovaries employed by Li and Gardner (*8*) were derived from mice of period 2, it was decided to transplant still older ovaries—from mice of period 4. Ovaries from weanling and young adult mice, both from period 1, also were transplanted.

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[2] The technical assistance of Frank M. McCathran is gratefully acknowledged.
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The primary object of this experiment was to observe whether old ovaries were susceptible to tumorigenesis and, if so, whether this differed from the susceptibility observed for younger ovaries. Tumors were obtained following transplantation of both young and old ovaries.

MATERIALS AND METHODS

C×C3H F_1 hybrid mice raised at the National Cancer Institute were employed as ovarian transplant donors and as hosts. These were maintained in an air-conditioned room with a temperature range of 76° to 78° F. and were housed in plastic cages 28 by 18 by 12 cm., no more than eight to a cage. Purina laboratory chow and tap water were supplied continuously in unlimited amounts.

Transplants were prepared as follows: Ovaries and Fallopian tubes were excised from donor mice, ages 29 to 855 days, immersed in sterile Locke's solution, and the gonads cleared of tubes and fat. The hosts, 67 to 220 days of age, were castrated immediately prior to transplantation. One-half an ovary, divided approximately into 1-mm. fragments, was transplanted intrasplenically to each host, using a No. 15G short-beveled trocar.

Mice were sacrificed generally 6 to 7 months after transplantation, when the presence of intrasplenic ovarian tumors was determined. All excised tissues were fixed in Tellyesniczky's fluid, sectioned at 8 micra, and stained with hematoxylin and eosin. Tumor diagnoses were confirmed histologically.[5]

EXPERIMENTAL PROCEDURES AND RESULTS

The mice were divided into three groups according to age of transplant.

In Series A, weanling ovaries were transplanted as follows: Twenty-nine-day old C×C3H F_1 hybrid ovaries to 25 C×C3H F_1 males 114 to 157 days old. The mean period of observation was 205 days.

In Series B, young adult ovaries were transplanted as follows: Series B_1 102- to 202-day old C×C3H F_1 hybrid ovaries to 21 C×C3H F_1 females 127 to 155 days old, and (Series B_2) 110- to 200-day old C×C3H F_1 hybrid ovaries to 22 C×C3H F_1 males 95 to 120 days old. The mean period of observation was 198 days for Series B_1 and 210 days for B_2.

In Series C, old ovaries were transplanted as follows: 690- to 855-day old C×C3H F_1 hybrid ovaries to 26 C×C3H F_1 males 68 to 196 days old. The mean period of observation was 221 days.

At autopsy, seven mice were observed with splenic adhesions to the body wall. These were excluded from the experiment and hence do not appear in the data in table 1.

In Series A, 21 of 23 males bore tumors, an incidence of 91.3 percent. The 2 nontumor-bearing mice were sacrificed 199 and 210 days following transplantation.

In Series B_1, 17 of 20 females, and in Series B_2, 18 of 20 males bore tumors, thus giving tumor incidences of 85 and 90 percent, respectively.

[5] The author is indebted to Dr. Thelma B. Dunn of the Laboratory of Pathology of the National Cancer Institute for her assistance.

Of the 5 nontumor-bearing mice, 2 were sacrificed 135 days, and 3, 210 days following transplantation.

In Series C, 16 of 24 males were observed with tumors, a tumor incidence of 66.7 percent. The 8 nontumor-bearers were sacrificed from 216 to 270 days following transplantation.

TABLE 1.—*Intrasplenic transplantation of weanling, young adult, and old ovaries from C×C3H F_1 hybrids into C×C3H F_1 hybrid castrates—all mice being gonadectomized at time of transplantation*

Series	Donor		Host					Ovarian tumors	
	Mean age of ovary		Mice in experiment—total [1]	Sex	Mean age of mice		Mean period of observation	Mice with tumors	Percent tumors
	At start	At autopsy			At start	At autopsy			
	Days	*Days*	*Number*		*Days*	*Days*	*Days*	*Number*	
A	29	234	23	♂	134	339	205	21	91.3
B_1	165	363	20	♀	142	340	198	17	85.0
B_2	129	339	20	♂	103	313	210	18	90.0
Entire series B	147	351	40	♀/♂	123	327	204	35	87.5
C	799	1,020	24	♂	128	349	221	16	66.7

[1] Mice with splenic adhesions to body wall not included.

From a comparison of the mean incidences of tumors for Series A, B, and C, the following Chi-square values were derived:

Series A compared with Series B: $X^2=0.36$; ($P>0.05$).

Series A compared with Series C: $X^2=6.1$; ($P<0.05$).

Series A+B compared with Series C: $X^2=7.08$; ($P<0.01$).

In these experiments, no attempt was made to study or to compare the various tumor types induced in ovaries from weanling, young adult, and senile mice. Granulosa-cell tumors, luteinized granulosa-cell tumors, luteomas, and mixed tumors containing both granulosa and lutein cells were observed. These have all been grouped together in the results. Although no detailed measurements of tumor size were made, large as well as small ovarian tumor masses were observed in all three of the series.

DISCUSSION

An average incidence of ovarian tumors of 66.7 percent was obtained following the intrasplenic transplantation of ovaries from senile mice. This provided an answer to the primary object of the experiment and showed that although spontaneous ovarian tumors are infrequent among old mice, ovaries from old mice remain susceptible and are capable of becoming neoplastic under suitable environmental conditions. Li and Gardner (*8*), previously reported the induction of ovarian tumors in aging ovaries. Although they reached no definite conclusions because of a small number of observations, it appeared that tumor incidence was about the same whether young or older ovaries were transplanted. In the present experiments, ovarian transplants were derived from senile mice

that averaged 799 days of age. On the other hand, the old ovaries transplanted by Li and Gardner (*8*) averaged 356 days in one series of four mice and 430 days in another series of four. Mice bearing senile ovarian transplants in the present experiments were sacrificed after a mean observation period of 221 days, whereas in the experiments of Li and Gardner autopsies were performed after an average of 356 days. In view of these differences in age of transplant, duration of experiment, and number of observations, it is difficult to compare the 66.7 percent tumor incidence we obtained with the 100 percent incidence reported by Li and Gardner (*8*). It is possible, however, that one or more of these variations may be responsible for the observed difference in tumor incidence.

It would appear that the low incidence of spontaneous ovarian tumors in old mice cannot be ascribed to a lowered susceptibility of old ovaries to pituitary gonadotrophin, since ovaries transplanted from old mice were susceptible in the present experiments as well as in those of Li and Gardner (*8*). However, fewer tumors were observed following the transplantation of old as compared with young ovaries, although the significance is borderline (table 1). Thus, in intact old mice, there is a suggestion that ovarian changes with age are partly responsible for the low incidence of ovarian tumors.

SUMMARY

Ovaries that averaged 29, 147, and 799 days, respectively, from C×C3H F_1 hybrid mice were transplanted intrasplenically into a total of 94 young C×C3H F_1 hybrid castrates. Corresponding to these ovarian age groups, average tumor incidences of 91.3, 87.5, and 66.7 percent, respectively, were obtained after a mean observation period of 198 to 221 days. Thus, while spontaneous ovarian neoplasms are infrequent in old mice, ovaries from old mice are susceptible but under a more favorable environment.

Old ovaries appear to be more resistant to tumorigenesis than young ones following intrasplenic transplantation, although the significance is borderline.

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FURTHER STUDIES ON METASTASIS OF A TRANSPLANTABLE MOUSE MAMMARY CARCINOMA AFTER ROENTGEN IRRADIATION [1] [2]

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INTRODUCTION

Kaplan and Murphy (*1*) have demonstrated that a significant increase in the rate of pulmonary metastasis occurs following local irradiation of the Bagg-Jacksen mouse mammary Carcinoma 755. Similar, though inconclusive, experimental evidence had previously been reported by two other investigators (*2, 3*). A number of possible mechanisms of this phenomenon were postulated, and this report is concerned with a series of experiments undertaken to test some of these.

MATERIALS

The Bagg-Jacksen mouse mammary Carcinoma 755 has been described previously (*1, 4*). No changes in appearance or biologic behavior of this tumor have been observed since the initial paper in this series.

Strain C57 black mice of both sexes were used in all experiments. They were identically caged and had free access to Purina laboratory chow and water.

Complete autopsies were performed on all experimental animals. Except where otherwise indicated, the lungs and other selected tissues were fixed in Bouin's solution, sectioned in paraffin, and stained in hematoxylin and eosin. In one experiment serial sections of all pulmonary lobes were made. Since metastases could be detected in many successive serial sections, it was considered satisfactory, in subsequent experiments, simply to obtain ribbon sections through three parallel longitudinal planes of each lobe.

EXPERIMENTS 1A AND 1B

In earlier experiments the tumor had been irradiated locally at the site of implantation on the hind leg, but the control procedure of locally ir-

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[2] Some of these experiments were conducted in the Laboratory of Biophysics, National Cancer Institute, Bethesda, Md.; others were performed in the Department of Radiology, Stanford University School of Medicine, with the aid of a grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

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radiating the contralateral hind leg had not been done. Such an experiment was therefore performed to learn whether local irradiation of the host alone during the phase of active tumor growth would also enhance metastasis. This experiment had to be repeated (1B) when, through a misunderstanding, different doses of irradiation were used over the two hind legs in Experiment 1A. Since the results are similar, however, both experiments are reported together.

Procedure

In Experiment 1A, 100 mice received tumor implants subcutaneously in the left hind leg. When the tumors had grown to approximately 1 cm. in diameter, half of the animals received local irradiation in a single dose of 3,000 r over the tumor-bearing area.[4] Most of the remaining animals similarly received local irradiation to the contralateral leg, but through error the dose was only 800 r. Ten animals received no treatment, either because their tumors grew very slowly or because of extraneous deaths.

In Experiment 1B, 121 littermate mice were randomized into 3 groups of approximately equal size. Tumor fragments were implanted into the dorsal subcutaneous space of the right hind leg in all groups. Approximately 2 weeks later, when the tumors were slightly under 1 cm. in diameter, mice of 1 group received a single dose of 800 r to the tumor-bearing area through a 1 cm. diameter aperture in a lead shield; another group received the same dose simultaneously through a similar aperture to the contralateral leg.[5] The third group was kept as untreated controls.

In previous experiments, pulmonary metastases have not been observed until 5 to 6 weeks after tumor implantation; mice dying before this interval were therefore not included in the final results although in Experiment 1B, all were autopsied, the lungs sectioned, and none found to have metastases. Mice dying 5 to 8 weeks after transplantation are designated as determinate. Survival time in the three groups was equalized in Experiment 1B by sacrificing moribund animals of two groups whenever a determinate death occurred in the third group. At the end of the determinate period, all survivors were sacrificed and autopsied as described above.

Results

The results of Experiments 1A and 1B are summarized in table 1. In Experiment 1A only three of the small number of untreated control animals survived into the determinate period, and none of these had metastases. The numbers of determinate survivors in the two groups of Experiment 1A are grossly unequal; this was largely prevented in Experiment 1B by the matched killing procedure described above. In both experiments, local irradiation over the tumor-bearing area elicited a significantly greater number of pulmonary metastases than were ob-

[4] Physical factors were: 186 KVP, 20 ma., 0.25 mm. Cu and 0.55 mm. Al added filter, focus-skin distance 30 cm., output 180 r per min.

[5] Physical factors were: 140 KVP, 20 ma., no added filter, H. V. L. 0.26 mm. Cu, focus-skin distance 40 cm., output 59 r per min.

TABLE 1.—*Incidence of metastases in experiment 1A and 1B*

Experiment No.	Group	Site of irradiation	Number of mice inoculated	Number of mice determinate	Mice with pulmonary metastases		σ [1]	Difference	t	p
					Number	Percent				
1A	1	Tumor	----------	47	10	21. 3	±5. 4	17. 0	3. 15	<0. 01
	2	Opposite leg	----------	23	1	4. 3				
1B	1	Tumor	39	33	15	45. 5	±7. 4	($p_1-p_2=$) 34.8	4. 7	<. 0001
	2	Opposite leg	40	28	3	10. 7		($p_1-p_3=$) 30.1	4. 1	<. 0001
	3	None	42	26	4	15. 4		($p_2-p_3=$) 4.7	. 63	. 33

[1] $\sigma=\frac{\sqrt{p_1 q_1+p_2 q_2+\ldots}}{n_1+n_2+\ldots}$

served in either untreated controls or in animals treated over the contralateral leg. The lack of a significant response in Group 2 of Experiment 1B militates against the hypothesis that the effect of irradiation is exerted locally on the host alone.

EXPERIMENT 2

It seemed of interest to determine whether the effect of irradiation in enhancing metastatic frequency partakes of the nature of a "somatic mutation." If this were true, the effect on the tumor would be expected to be permanent, and it could be anticipated that metastases so induced would, on reimplantation, continue to exhibit a rate of metastasis significantly greater than that of the original tumor.

Procedure

In three separate experiments, small groups of mice bearing tumor 755 were locally irradiated over the tumor with single doses of 1,000 r; after observation, mice with distinct gross metastases were then used for the experiment.[6] Lungs containing metastatic nodules were excised and the nodules were cut into small fragments for reimplantation. The reimplanted tumors received no further irradiation. Because the implanted fragments were smaller than those usually used, a longer time was required for the tumors to attain equivalent size. Animals were observed for over 2 months, and all were examined at autopsy for evidence of pulmonary metastases.

Results

The results are summarized in table 2. No metastases were observed in any of these three small groups of mice. This suggests that there is no permanent alteration in the biologic behavior of tumor 755 after irradiation, rather that the augmentation of metastatic frequency results from a transient disturbance in the host-tumor relationship.

Table 2.—*Incidence of metastases in experiment 2*

	Experiment 2 (a)	Experiment 2 (b)	Experiment 2 (c)	Total
Number of mice inoculated	11	10	3	24
Number determinate survivors	9	10	3	22
Number with metastases	0	0	0	0

EXPERIMENT 3

The purpose of this experiment was to determine whether the effect of irradiation on metastatic frequency could be elicited by local irradiation of the host alone, or of the tumor either *in situ* in a previous host, or *in vitro*.

[6] Physical factors were: 120 KVP, 9 ma., 0.25 mm. Cu and 1 mm. Al added filter, H. V. L. 0.36 mm. Cu, focus-skin distance 21 cm., output 64 r per min.

Procedure

There were four separate experimental groups, which were implanted with tumors from four littermate mice of the same transplant generation.

Group (a). In vivo irradiation of tumor in previous host.—A subcutaneously growing tumor mass 4.5 by 2 by 2 cm. received a single dose of 1,000 r locally, with the remainder of the host animal shielded in a special brass holder.[4] About 20 hours later the mouse was killed, the tumor was excised, and fragments were implanted subcutaneously on the left hind legs of 50 nonirradiated mice.

Group (b). Untreated controls.—A tumor measuring 3 by 2.5 by 2 cm. was excised from a littermate mouse of the same transplant generation, and fragments were implanted subcutaneously in the left hind legs of 36 nonirradiated mice.

Group (c). Irradiation of host alone.—Forty mice were locally irradiated with a single dose of 1,000 r over the rump and hind-leg area.[4] About 24 hours later, fragments from a freshly excised, nonirradiated tumor of the same transplant generation were implanted subcutaneously into the irradiated area.

Group (d). In vitro irradiation of tumor.—The tumor mass from a fourth mouse of the same transplant generation was excised, placed in a petri dish on a filter paper moistened with cold saline solution, and exposed to a single dose of 1,000 r.[4] Immediately thereafter, it was cut into fragments that were implanted subcutaneously into the left hind legs of 30 untreated mice.

Results

The results are summarized in table 3. The incidence of metastasis was low in all groups and, although no metastases occurred in the control group in this particular experiment, the percentage of mice with metastases in the other groups did not exceed that observed in several previous control groups and was not considered significant. It is concluded that the frequency of metastasis is not increased by irradiation of either the host or the tumor alone.

Table 3.—*Incidence of metastases in experiment 3*

Group	Irradiation	Number of determinate mice	Mice with metastases	
			Number	Percent
(a)	Tumor in previous host	39	3	7.7
(b)	None	32	0	
(c)	Host alone	35	5	14.3
(d)	Tumor, *in vitro*	23	2	8.7

EXPERIMENT 4

Casey (*5, 6*) has described an "XYZ" material obtainable after anaerobic autolysis of several transplantable tumors, which is capable of enhancing the rates of growth and metastasis of the homologous tumors. It was considered possible that a similar autolysate might be produced *in vivo*

by local irradiation of tumor 755 and that its release might stimulate the occurrence of metastasis.

Procedure

Tumors freshly excised from several mice of the same transplant generation were pooled in large test tubes, covered with a layer of paraffin, and set in the refrigerator for approximately 3 months. A thick semiliquid material accumulated at the upper end of the test tube and was drawn off at that time. Forty mice were injected subcutaneously with 0.3 cc. of this material 9 days prior to subcutaneous implantation of tumor fragments in the usual manner. An additional 20 mice were implanted with the tumor but received no injection of the "XYZ" material. Animals were observed for several weeks, autopsied, and examined for metastases in the usual manner.

Results

Of 34 determinate mice in the "XYZ"-injected group, only 1 exhibited pulmonary metastases. There were 15 determinate mice in the control group, of which one had metastases. It is therefore concluded that under the conditions of this experiment, an anaerobic autolysate of tumor 755 failed to reproduce the effect of local irradiation on metastatic frequency of this tumor.

EXPERIMENT 5

The purpose of this experiment was to learn whether nonirradiated tumor cells artificially introduced into the blood stream were capable of growing as "metastases" in the lung. The possibility was also considered that the presence of a tumor mass growing subcutaneously might condition the ability of tumor cells to "take" in the lung.

Procedure

Preliminary studies were conducted to determine the viability of tumor-cell suspensions made in various ways. A modification of the method of Zeidman *et al.* (7) was found to be satisfactory. A large freshly excised tumor was forced through a fine metal sieve into cold Locke's solution. The decanted suspension was examined by dilution in a white blood-cell pipette with cold dilute trypan blue in Locke's solution and counting in a standard hemocytometer. In freshly prepared suspensions, most of the cells occurred singly although clumps of 2 to 10 cells were numerous. About 30 percent of the cells appeared to be viable as judged by lack of dye uptake and sharpness of cellular outlines.

A total of 102 littermate mice was randomized into 3 groups, of which 2 were subcutaneously implanted with tumor fragments in the usual manner, and the third was kept intact. Beginning about 3 weeks later, mice of one of the tumor-bearing groups and the intact group received intravenous injections of a suspension of tumor cells. Each mouse received 0.5 cc. of the suspension, injected slowly through a tuberculin syringe and 26-gauge needle into the tail vein. Immediately following each series of injections a cell count was made on each suspension. The

calculated number of cells injected per mouse ranged from 11,700 to 3,750,000. Fresh tumors were used for all cell suspensions, and an equal number of mice from each of the 2 groups was injected with each suspension thus prepared. Microscopic sections of the lungs of 2 mice dying immediately after injection revealed clumps of tumor cells in the small pulmonary blood vessels.

Two other mice were transplanted through transperitoneal incisions: in one, tumor cells in suspension were injected directly into the spleen and liver, while in the other a tumor fragment was inserted through a sharp trocar into the spleen. Since intrasplenic and intrahepatic metastases had not previously been observed, it seemed of interest to determine whether the tumor was capable of growing in these sites if artificially introduced into them.

Mice dying less than 5½ weeks after transplantation have been considered indeterminate and excluded from the tabulated results. These mice were, however, autopsied and no pulmonary metastases were detected in them. The lungs of determinate animals were screened for metastases by *in toto* staining with borax-carmine, followed by differentiation in 0.01 percent HCl. All lobes were then cleared in cedarwood oil and examined under a 40× binocular dissecting microscope. Metastases appeared as relatively avascular, lightly staining nodules against a background of darker-staining, highly vascular pulmonary tissue. The nodules were visibly lobulated, occasionally clearly intravascular, and often peripheral in location. All positive and suspicious pulmonary lobes were sectioned and stained in the usual manner for histologic confirmation. There were many initial false positives due to pneumonia but the screening procedure effectively detected several very minute metastases.

Results

The results are summarized in table 4. Standard errors calculated from the formula $\sigma=\sqrt{p^q/_n}$ yielded values of ± 5.33 for the pooled data of the injected Groups 1 and 2, which on comparison with the uninjected Group 3 gave $t=4.4$, $P<0.0001$. Omitting Group 2, comparison of Groups 1 and 3 gave a standard error for Group 1 of ± 6.2, $t=2.4$, $P<0.05$, >0.01. It is therefore concluded that the intravenous injection of tumor cells in suspension led to the development of pulmonary tumor nodules more often than in uninjected animals bearing the nonirradiated subcutaneous tumor.

TABLE 4.—*Incidence of metastases in experiment 5*

	Tumor plus injection	Injection only	Tumor only	Total
Number inoculated	36	33	33	102
Determinate survivors	33	30	23	86
Number with metastases	5	10	0	15
Percent determinate survivors with metastases	15	33	0	----

The appearance of some of the pulmonary tumor foci in the injected mice differed from that of the spontaneous or radiation-induced pulmonary metastases previously observed. Grossly, they were usually 1 to 2 mm. in diameter, sessile or pedunculated, grey, opaque growths. Although sometimes single, more commonly there were two to three nodules scattered among the pulmonary lobes. Microscopically, there was often a marked round-cell reaction in and about these nodules. The foci were often peripheral and less commonly intravascular as in the case of radiation-induced metastases. In Group 1, however, four of the five mice with pulmonary tumor foci had typical intravascular central "metastases" without round-cell reaction; the other showed a single peripheral nodule surrounded by round cells.

Tumor cells injected directly into the spleen and liver of one mouse grew readily, and the abdomen was found to be distended within 2 weeks. Similarly, in the mouse bearing the intrasplenic tumor implant, the spleen was largely replaced with a tumor 3 by 1 by 1 cm. within 2 weeks after implantation. The capsule of the spleen remained intact but numerous small metastases to the liver were observed. No pulmonary metastases were seen in either animal. It would appear, therefore, that lack of metastases in the liver and spleen after local irradiation of tumor 755 is not attributable to any inability of the tumor to grow in these sites; instead, it seems likely that tumor cells are effectively filtered from the circulation by the lung, thus accounting for the exclusively pulmonary localization of metastases that have hitherto been observed.

DISCUSSION

The results of this series of experiments are helpful in narrowing down the number of possible mechanisms that still remain to be investigated. It appears that the possibility of a "somatic mutation" is effectively excluded (Experiment 2). The negative outcome of Experiment 4 militates against, but does not completely exclude, the possibility that an autolysate similar to Casey's "XYZ" factor may result from local irradiation of tumor 755 and act as an intermediate agent in producing pulmonary metastases. It is possible that the method used for preparing the autolysate in this single experiment was not optimal, or that the interval between injection of the autolysate and implantation of the tumor was unsuitable. The methods used were, as far as possible, identical with those described by Casey (*6*).

In Experiment 3, it was found that irradiation of neither the host alone, nor of the tumor alone, is capable of eliciting an increased frequency of pulmonary metastasis. This fact, together with the results of Experiment 2, supports the view that the effect involves a transient disturbance of the host-tumor relationship.

Of importance also is the demonstration (Experiment 5) that the cells of tumor 755, when injected intravenously to facilitate direct passage to the lung, are readily capable of progressive growth in pulmonary tissue without previous irradiation. While this does not exclude the possibility

that irradiation enhances the ability of the tumor cells to grow in the lung, it seems unnecessary to postulate such an effect.

It would appear, therefore, that future investigations should focus on *local* disturbances following irradiation of the subcutaneous tumor *in situ*. It is well known that damage to blood vessels, with increase in capillary permeability, may result from irradiation (*8*, *9*). It seems plausible that tumor cells might more easily penetrate vessels that have thus been damaged and then be carried to the lung. Mention should also be made of the possibility that irradiation may further decrease the mutual adhesiveness of tumor cells (*10*) or affect their ameboid motion (*7*) and thus their ability to reach and enter blood vessels.

SUMMARY

A series of five experiments concerned with the frequency of pulmonary metastasis in strain C57 black mice bearing the Bagg-Jacksen mouse mammary Carcinoma 755 are reported.

Experiment 1 confirmed the earlier report of Kaplan and Murphy (*1*) that pulmonary metastases occur more frequently after local irradiation of this tumor than in nonirradiated controls. There was no such effect when the host was locally irradiated in a nontumor-bearing area.

Experiment 2 demonstrated that the metastases thus induced do not possess a permanently increased capacity for subsequent metastasis and thus that the effect is probably not a "somatic mutation."

Experiment 3 revealed that irradiation of neither the host alone nor of the tumor alone is capable of yielding this effect.

Experiment 4: an anaerobic autolysate similar to Casey's "XYZ" factor injected into mice prior to tumor implantation failed to stimulate metastasis.

Experiment 5: suspensions of viable cells of tumor 755 injected intravenously into mice grew to form "metastatic" nodules in the lungs.

It is concluded that the effect of irradiation on metastasis of this tumor involves a transient local disturbance of the host-tumor relationship, probably concerned with facilitating entry of tumor cells into blood vessels.

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PROLIFERATIVE LESIONS RESEMBLING CHORDOMA FOLLOWING PUNCTURE OF THE NUCLEUS PULPOSUS IN RABBITS [1]

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Ribbert was the first to study the results of puncturing the nucleus pulposus in laboratory animals, and the present paper is largely a confirmation and extension of his work (*1*). His attempts to transplant the nucleus pulposus to the anterior chamber of the rabbit's eye failed, and he turned to the lesion resulting from merely puncturing the nucleus pulposus in an attempt to confirm his theory of origin of the human chordoma. Within 5 days after the puncture Ribbert found regeneration of nucleus pulposus cells as indicated by the presence of numerous mitoses. There was progressive growth of the extruded nucleus pulposus cells for several months, but by the end of a year retrogression of the lesion had occurred. Ribbert concluded that the lesion was histologically identical with the ecchordosis physaliphora found along the clivus in humans and described earlier by Luschka (*2*), Virchow (*3*), and others (*4*). Ribbert interpreted the lesion as substantiating his belief that chordoma develops from misplaced adult cells rather than from misplaced embryonic cells as Cohnheim believed.

Keyes and Compere (*5*), in their extensive study of the nucleus pulposus, reported the lesions produced in monkeys and adult dogs by injuring the intervertebral disk in different ways. Loss of the disk with osteoarthritic changes and a kyphosis developed in the dogs that had had curettement of the nuclear material of the intervertebral disk. When small drill holes were made through the vertebral bodies into the nucleus pulposus, herniation of the latter into the vertebral body produced a lesion similar to a Schmorl's nodule. In the cases in which puncture was carried out, with escape of the nuclear content through the annulus fibrosus, narrowing of the intervertebral-disk space occurred. No mention was made of proliferative changes in the nucleus pulposus cells in these adult animals.

In 1932 Schrader (*6*) reported production of spondylosis deformans in rabbits following puncture of the intervertebral disk. No soft-tissue changes were described in the preliminary report.

Lob (*7, 8*) in 1933 described the macroscopic and roentgenologic findings in an extensive experimental study in rabbits, involving injury to the

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intervertebral disk by electrical current, incision, and chemical agents. Lob was concerned primarily with the vertebral lesions similar to spondylosis deformans that resulted from injury to the disk. He apparently was of the opinion that the nucleus pulposus cells gave rise to cartilage or remained in the injured areas as cell rests. The changes described in these cells in some of the animals resembled those described by Ribbert (*1*) and found, also, in the present work.

Tammann (*9*) in 1934 reported the results of injury of the intervertebral disk in dogs after intervals of 2 weeks, 5½ months, and 6 months. The age of the dogs was not mentioned. He found the remains of the nucleus pulposus in the wounds but no evidence of its regeneration. As with other workers, his interest was in the vertebral lesions resembling spondylosis deformans.

A study of regeneration of the disk by fibrocartilage was reported by Filippi (*10*) in 1935. Observations in rabbits, whose disks had been punctured anteriorly from 10 to 100 days previously, showed no remnants of the nucleus pulposus and no evidence of its taking part in the fibrocartilaginous regeneration of the disk. The age of the animals was not given, and his figures showed that the anterior portion of the intervertebral disk had been shaved off smoothly.

Spontaneous chordoma has been reported in the dog and the rat. Ball and Auger (*11*) reported a poorly circumscribed chordoma, 6 by 3 by 4 cm., originating in the cervical vertebral column of a 6-year-old cocker Spaniel dog. It recurred rapidly after surgical excision, and the recurrence did not respond to X-ray therapy. A lumbar chordoma was described by Morpurgo (*12*) in a rat with osteomalacia.

METHOD AND MATERIALS

Fifteen stock rabbits about 8 weeks of age were subjected to laparotomy under ether anesthesia. After packing aside the intestines to secure exposure of the aorta and psoas muscles, the lumbar intervertebral disks were located by palpation. The disk to be punctured was then exposed by splitting the muscles with a large hemostat. The procedure was usually bloodless but occasionally oozing was encountered. A small dental chisel was used to puncture the disk. As the chisel was removed the gelatinous contents composing the nucleus pulposus squeezed out through the defect, forming a small mound about 3 mm. in diameter on the anterior surface of the intervertebral disk. The muscles were allowed to fall into place over the disk, and the abdominal wound was closed. In six animals two disks were punctured.

In two animals (Nos. 22 and 23) the extruded nucleus pulposus was removed by suction and transplanted to the subcutaneous tissues of the abdominal wall.

An attempt was made in two other cases (Nos. 13 and 24) to introduce 20-methylcholanthrene into the nucleus pulposus. In rabbit No. 13 a portion of a 10.2-mg. pellet of 20-methylcholanthrene was pushed into the nucleus pulposus. During the process most of the nuclear content

was extruded. A knotted thread dipped in melted 20-methylcholanthrene was pushed into the nucleus pulposus in rabbit No. 24. No extrusion of the nucleus pulposus was noted at the time of the operation.

The animals were killed at intervals of 99 to 319 days following the operation. The portions of the vertebral column including the injured disks were dissected and fixed in formalin. Hematoxylin and eosin sections of the lesions were prepared. In rabbit No. 24 metachromatic staining was carried out before and after incubating a paraffin section with hyaluronidase to demonstrate the presence of connective-tissue mucin.[2]

RESULTS

Table 1 gives the results obtained in this series. For convenience in analysis, the animals were listed in ascending order as to elapsed time between perforation of the disk and sacrifice.

In 3 to 4 months an easily recognized nodular enlargement of the anterior surface of the intervertebral disk had developed, with small nests of vacuolated nucleus pulposus cells covering the surface. In rabbits Nos. 12 and 2 some of the nests of these cells were growing on the posterior surface of the disk but in the remaining 5 animals examined after this interval growth of cells of the nucleus pulposus was entirely anterior.

The nodular enlargement of the disk varied from 1 to 2 cm. in diameter and was composed of new-formed bone and cartilage growing out of the cartilage and bone plates of the adjacent vertebral bodies. In some cases small nests of nucleus pulposus cells were trapped in the cartilaginous masses, and the bony part of the enlargement showed blood-forming elements in marrow spaces. Figures 1, 2, 3, and 4 show the gross features of the lesions. Figure 5 shows nests of the vacuolated nucleus pulposus cells on the surface of a disk. These nests varied greatly in size, ranging from a few cells to grossly visible nodules. Most of the cells had a large vacuole distending the cell, and usually a small, round, dark-staining nucleus was visible. An occasional nest had nonvacuolated cells with a scant pink cytoplasm, and then intercellular mucin was present, giving a blue-staining matrix for the cell nest. In some nests nuclei had disappeared, indicating that retrogressive changes were already under way.

Rabbit No. 13 of this group had a large nodule of regenerating nucleus pulposus cells in the psoas muscle. This animal, which had had a pellet of 20-methylcholanthrene placed in the disk, was the only one in this group to show muscular involvement.

The punctured intervertebral disks usually showed narrowing due to loss of the nucleus pulposus. In some animals in which a large part of the nucleus pulposus was not extruded, the disk showed no differences from normal disks in the same animal. The cortical bone adjacent to the disk on both sides became much thicker as did the cartilage plates. In rabbit No. 13, which received 20-methylcholanthrene, the disk was

[2] This procedure was kindly carried out by Dr. Arthur C. Upton.

TABLE 1.—*Summary of experimental procedures and results*

Animal No.	Duration of experiment (days)	Number of disks punctured	Additional procedures	Gross involvement of psoas muscles	Microscopic involvement of disk surface by nucleus pulposus cells	Intervertebral disk changes
13	99	1	20-methylcholanthrene pellet into disk.	1 cm. nodule in muscle.	Large nests of cells on anterior surface of disk.	Partial destruction of disk and chronic inflammatory changes in adjacent bone.
14	99	2	----	None	2 small nests of cells in relation to one disk surface.	Partial disruption of an intervertebral disk by severe chronic inflammatory process.
12	106	1	----	do	Small nests of cells along anterior surface of disk. Some show loss of nuclei. Slight protrusion into spinal canal.	Part of nucleus pulposus space remains and contains normal cells. Nodule of cartilage and bone on anterior surface of disk.
1	112	1	----	do	Large mass of cells filling puncture wound. Small nests along disk surface.	Loss of nucleus pulposus space. Nodule of cartilage and bone replaces anterior surface of disk.
4	116	1	----	do	Large and small nests along anterior surface of disk. Small nests enclosed by cartilage.	Cartilaginous and bony nodule replaces disk surface.
2	121	1	----	do	Small nests of cells along posterior surface of the intervertebral disk and adjacent to spinal meninges.	Loss of nucleus pulposus space. Cartilaginous nodule on posterior disk surface. Bone and cartilage nodule on anterior disk surface.
6	121	1	----	do	Small nests along anterior surface of disk.	Narrowing of disk. Cartilaginous and bony nodule at disk surface.
24	182	1	Thread with 20-methylcholanthrene into disk.	2 × 2 cm. nodule in muscle.	do	Cartilaginous and bony nodule at disk surface.
23	190	2	Transplantation of nucleus pulposus.	None	Small nests of cells on surface of both disks.	Partial loss of nucleus pulposus spaces. Nodule of bone and cartilage on surfaces of both disks.
19	191	2	----	Small nodule in muscle.	Small nest of cells(?) on surface of 1 disk.	Small nodules of bone and cartilage on disk surfaces. Loss of nucleus pulposus spaces.
18	192	2	----	0.5 cm. nodule in muscle.	Small nests of cells along anterior surfaces of the 2 disks.	Loss of nucleus pulposus spaces. Small nodules of cartilage and bone make up anterior surface of both disks.

22	317	1	Transplantation of nucleus pulposus.	None	Many large and small nests of cells on anterior surface of disk. Some show retrogressive changes.	Loss of nucleus pulposus space. Bony nodule replaces disk surface.
20	318	2	----	1.5-cm. nodule in muscle.	Nests of cells along 1 disk surface	Nodule of bone and cartilage on anterior surface of one disk.
21	318	1	----	Small nodule in muscle.	Many large and small nests on anterior surface of disk.	Bony nodule replaces anterior surface of disk. Loss of nucleus pulposus space.
17	319	2	----	2.5 × 0.5 cm. nodule in muscle.	Many nests of cells on anterior disk surfaces. Some show retrogressive change with loss of nuclei. Others are proliferating.	Loss of nucleus pulposus space. Anterior surface shows no microscopic alterations except for nucleus pulposus cells.

destroyed and an extensive inflammatory reaction was present, with collapse of the adjacent vertebral bodies.

The eight animals that lived from 6 to 10½ months after puncture of the intervertebral disks showed essentially the same changes as the earlier group. The nodular enlargement at the anterior surface of the disk was greater, as seen in figures 3 and 4. With the exception of Nos. 22 and 23, these animals all showed gelatinous nodules of nucleus pulposus cells visible grossly in the psoas muscle adjacent to the punctured disk. (These two animals had had the nuclear contents removed and transplanted to the abdominal wall.) The nodules in the muscle varied from 0.5 cm. to 2.5 cm. in diameter (fig. 6). In general, their histologic structure was similar to that of the nests on the disk surface. In areas retrogressive changes with loss of nuclei were seen. The proliferative activity of the living cells was indicated by hyperchromatism of nuclei, presence of multinucleate cells, absence of vacuoles in the cytoplasm of some cells, and by growth between muscle fibers in certain instances. In the two animals in which transplantation of the nucleus pulposus was attempted, no trace of the transplant could be found in the abdominal wall.

The most striking lesion of the entire series was that of rabbit No. 24 in which a thread impregnated with 20-methylcholanthrene had been pushed into the nucleus pulposus. The nucleus apparently leaked out through the annulus of the disk after the laparotomy and a 2 by 2 cm. gelatinous nodule formed in the psoas muscle. Histologically this had all of the characteristics of a benign chordoma. Figure 7 shows the slight cellular pleomorphism. This lesion failed to stain metachromatically after incubating a paraffin section with hyaluronidase, indicating the presence of connective-tissue mucin. The pre-incubation section showed marked metachromatic staining. A section of a malignant chordoma from a human case showed an identical reaction.

DISCUSSION

It is generally agreed that the anlagen of the nuclei pulposi are the vacuolated cells of the notochord. In this paper the term nucleus pulposus cells indicates these elements as they are found in the nucleus pulposus. In man these cells disappear from the nucleus pulposus after the seventh year of life, except in occasional individuals and in those who develop benign and malignant chordomas. Their persistence along the notochordal tract outside the nucleus pulposus is well known.

The life cycle of the nucleus pulposus cells in the rabbit has not been studied, but there is suggestive evidence that retrogressive changes occur with aging in the nucleus pulposus of this animal, and that a loss of these cells occurs in the rabbit as in man. Some workers failed to find regeneration of nucleus pulposus cells, but this may have been due to the age of the rabbits they used and to the fact that they paid particular attention to the spondylitic changes in adjacent vertebrae.

The regenerative process following release of the nucleus pulposus as originally described by Ribbert (*1*) has been reproduced in the course of these experiments. Both from the mechanical aspects of its production and from the extent and character of the subsequent proliferation, this process appears to be unique in the field of regenerative changes. For most of the supportive tissues, mechanical stress or pressure such as weight-bearing promotes regeneration and progressive differentiation. This principle is constantly applied in corrective surgery. For the nucleus pulposus, however, it appears that release from constrictive pressure promotes regenerative proliferation.

The nodular masses of nucleus pulposus cells that developed following puncture of the disks exhibited some of the attributes of true neoplasms. Some nodules grew to a volume many times greater than that of a normal nucleus pulposus, there was evidence of local infiltrative growth, and they served no conceivably useful purpose in the economy of the animal. Proof of potentiality for unlimited growth was not obtained, although one of the larger masses was found in rabbit No. 17, killed 319 days after puncture. Discontinuous growth beyond the general area of operative trauma was not observed.

The tissue response in the two animals that received a chemical carcinogen in addition to mechanical trauma proved of special interest. These animals showed earlier and larger growths than others. This procedure was undertaken as a pilot experiment only, and the number of animals does not justify a firm conclusion; but it is evident that a combination of puncture of the disk and the introduction of a carcinogen such as 20-methylcholanthrene offers promise as a means of producing a neoplasm resembling the human malignant chordoma.

SUMMARY

The regeneration of nucleus pulposus cells following puncture of the intervertebral disks of rabbits, as described by Ribbert, has been confirmed. The resulting growths present some, but not all, of the features of true neoplasms.

Two animals that received local applications of 20-methylcholanthrene in addition to puncture showed earlier and more extensive lesions than those with puncture alone. The possibility of experimental production of a malignant chordoma in the rabbit is suggested by this work.

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PLATE 82

FIGURE 1.—Nodular enlargement of the intervertebral disk in rabbit No. 12, 106 days after puncture. The adjacent disks are normal. × 2

FIGURE 2.—A sagittal section through the lesion shown in figure 1. × 2

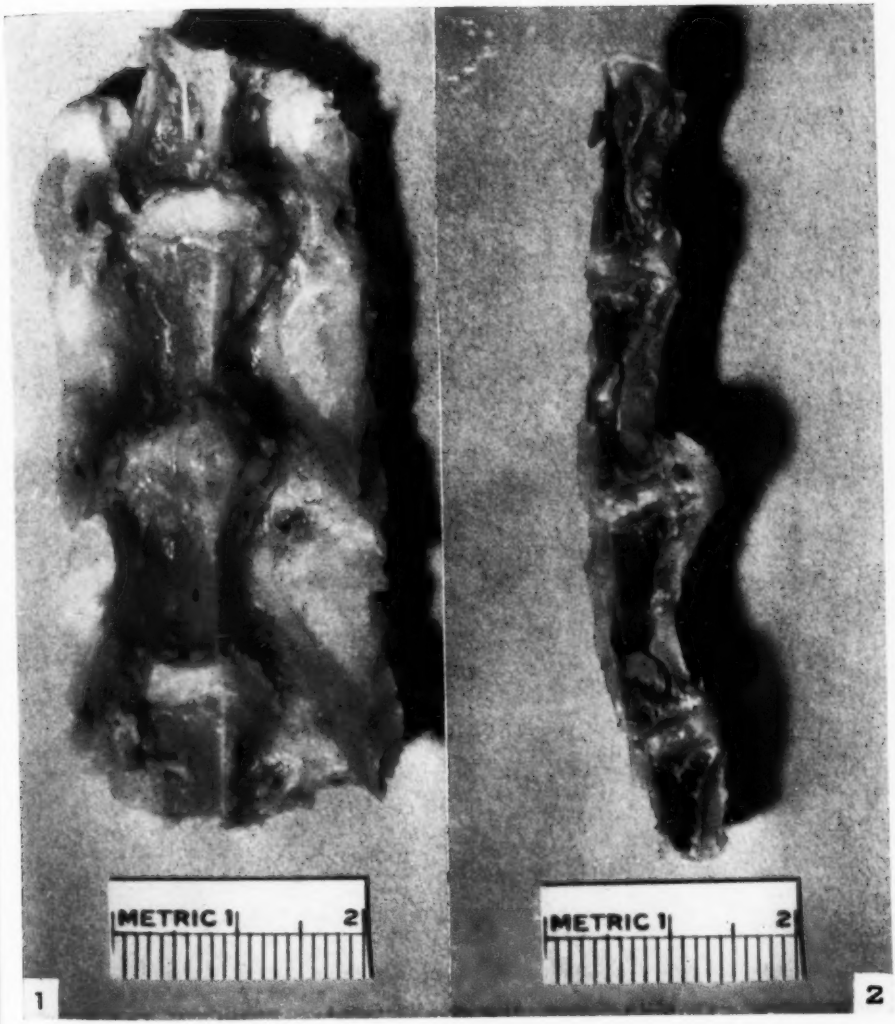

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PLATE 83

FIGURE 3.—Rabbit No. 12. Intervertebral disk lesion, 106 days after puncture. × 2

FIGURE 4.—Rabbit No. 17. Two intervertebral disk lesions, 319 days after puncturing. × 2

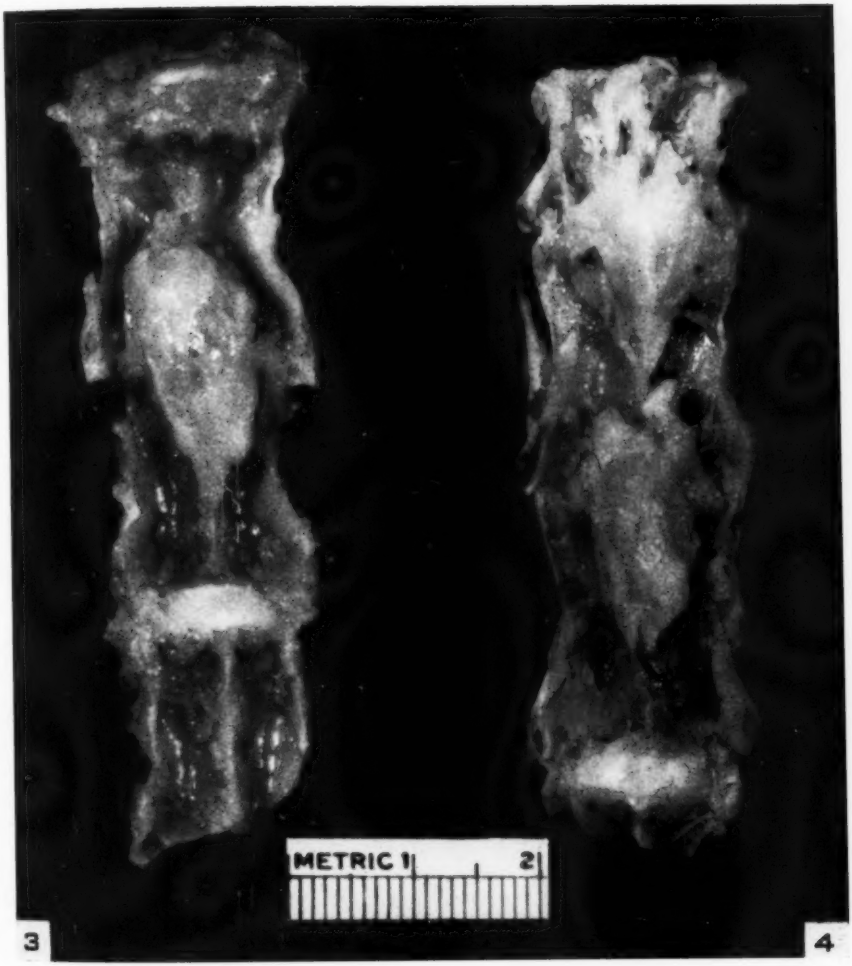

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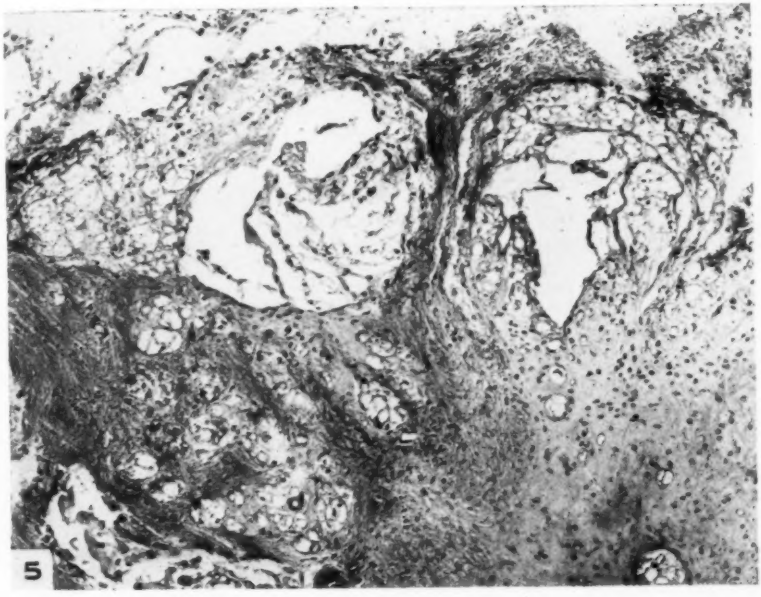
PLATE 84

FIGURE 5.—Nests of nucleus pulposus cells along the anterior surface of the intervertebral disk of rabbit No. 4, 116 days after puncture. × 100

FIGURE 6.—The muscle lesion in rabbit No. 18, 192 days after puncture. × 100



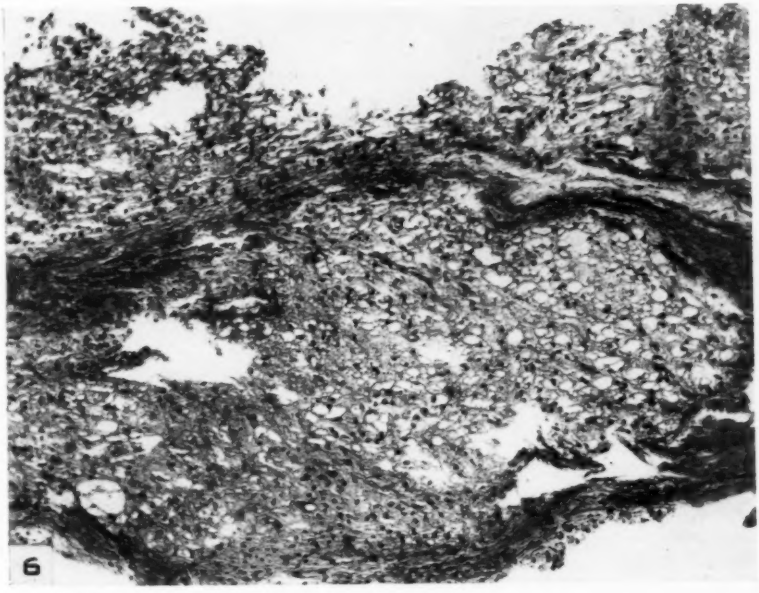


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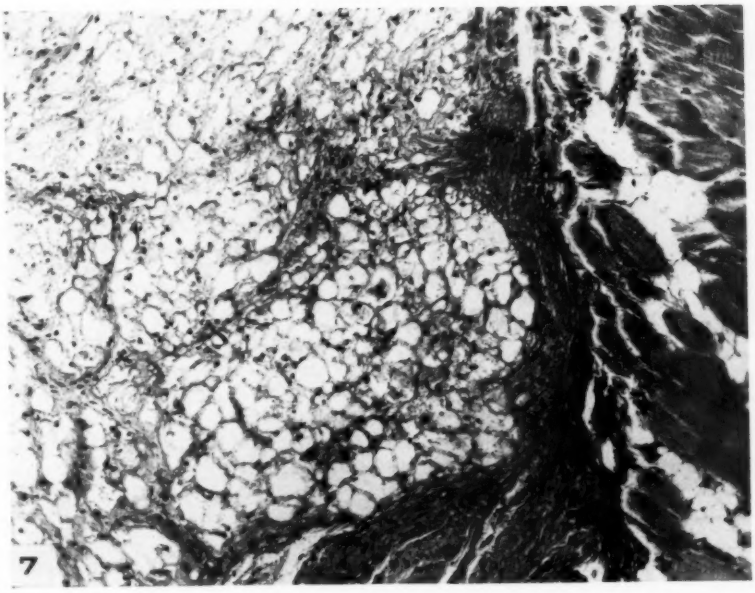
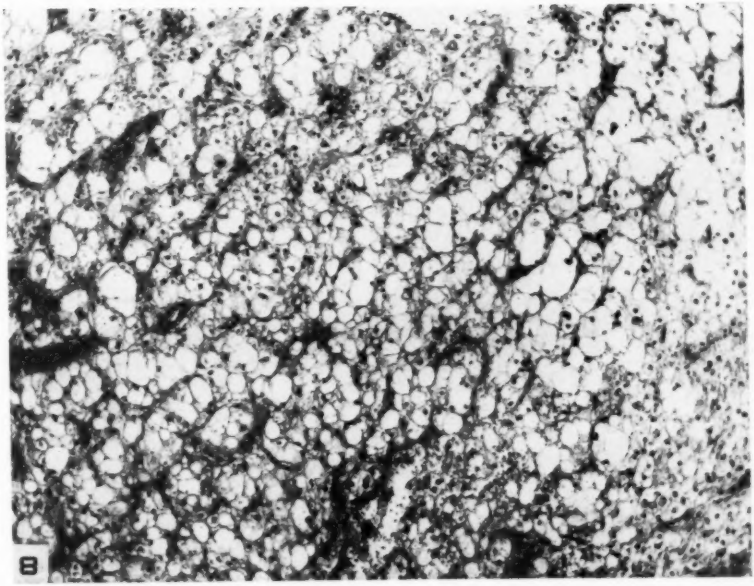
PLATE 85

FIGURE 7.—Rabbit No. 24. Lesion that developed at the site of extrusion 182 days after placing a thread impregnated with 20-methylcholanthrene into the nucleus pulposus. × 100

FIGURE 8.—A biopsy specimen taken from a 53-year-old woman who died of a sacral chordoma after a 7-year illness. The area photographed was picked because of the resemblance to the growths produced in the rabbit. × 100





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INHERITANCE OF LOW LIVER GLUCURONIDASE ACTIVITY IN THE MOUSE [1]

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In previous studies it was reported (*1,2*) that the liver glucuronidase activity of several sublines of C3H mice, a strain with a high incidence of hepatomas and mammary tumors, was approximately one-tenth that observed among a number of other well-known inbred mouse strains, *viz.* A, Strong CBA, C57 black, B alb C, DBA, RIII, and I. Among the other strains tested only the Andervont subline of the strain CBA, which like the Strong CBA subline is closely related in descent to the strain C3H, manifested a reduced level of liver glucuronidase activity. A relative reduction of glucuronidase activity in the spleen and kidney, less marked than in the livers, was also observed among C3H mice. No differences in the pH maxima and other characteristics of the enzyme were found by comparison of C3H livers with those from the strains exhibiting high levels of glucuronidase activity. There was no relationship of the mammary tumor milk-agent to the liver glucuronidase activity. In view of the uniformly low glucuronidase levels observed in C3H animals obtained from three different breeding sources, the investigation of the inheritance of liver glucuronidase appeared to be warranted.

MATERIALS AND METHODS

The nucleus of the strain A mice used in this study comprised 2 litters of the 82d and 83d inbred generations, which were obtained from Dr. W. E. Heston of this Institute. Strain C3H mice were of the 19th and 20th inbred generation.[4] The production of mice for glucuronidase assays proceeded from these animals.[5]

Matings were made reciprocally between the 2 strains to produce the F_1 hybrid generation, there being 7 strain A females mated to 2 strain

[1] Received for publication November 16, 1951.

[2] With the technical assistance of Dorothy M. Carroll.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

[4] The strain C3H was obtained originally by Dr. W. E. Heston from Dr. H. B. Andervont of this Institute. F_{19} and F_{20} indicate the number of generations of continued brother × sister matings since that time.

[5] A small number of mice of the parental strains, F_1 hybrids and the C3H backcross, were obtained from Dr. J. J. Bittner and are included in the combined data. Mean glucuronidase activities were strikingly similar to values obtained with our mice.

C3H males and 7 strain C3H females mated to 2 strain A males. The strain A mice were brothers and sisters but the strain C3H mice were from 2 different litters of the F_{19} and F_{20} generations. Sixteen females and 3 males from the A × C3H matings were used to produce an F_2 and 2 backcross generations. From the C3H × A matings 5 males were used to produce F_2 and backcross generations. The various matings may be summarized and designated for convenience as follows:

C3H × C3H

A × A

F_1=(A × C3H) and (C3H × A)

F_2=(A × C3H) × (A × C3H) and (C3H × A) × (C3H × A)

C3HBC=(Backcross to C3H)=[C3H × (C3H × A)] and reciprocal mating.

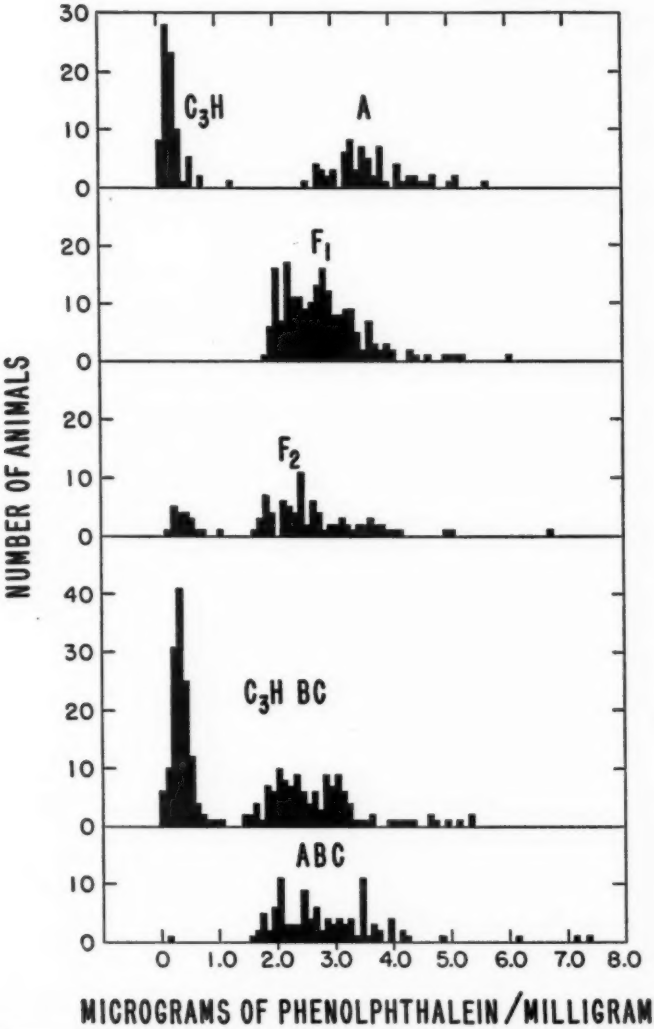
ABC=(Backcross to A)=[A × (C3H × A)] and reciprocal mating.

In addition to the above crosses we have utilized the method of individually classifying various members of the C3HBC cross, the backcross to the strain C3H, in order to determine the manner of segregation of factors possibly influencing low glucuronidase activity. Eighteen C3HBC backcross males were partially hepatectomized at 60 days of age, and the liver glucuronidase activities determined. Each of these males was mated in a second backcross to 5 C3H females. Only 14 of these backcross males produced offspring, and in some crosses only relatively few mice were obtained. This second backcross of C3H backcross males [C3H × (A × C3H)] to strain C3H females is designated C3HBC2 (low) for matings using a backcross male with a glucuronidase activity below 1γ/mg., and C3HBC2 (high) for matings with backcross males having glucuronidase activities greater than 1γ/mg. of moist liver tissue. The majority of C3H females used in this cross were assayed for glucuronidase activity (liver) following their use as breeders. The liver glucuronidase levels obtained were all low, characteristic of the strain, and varied from 0.10 to 0.87 γ/mg.

Mice of the various crosses were weaned at 4 weeks of age, segregated according to sex, and maintained under normal laboratory conditions until the age of 60 days (17–25 grams) when glucuronidase assays were accomplished. Glucuronidase determinations were performed on uncentrifuged tissue of homogenates, using a modification of the method of Talalay (*3*) and employing phenolphthalein glucuronide as a substrate. The details of this procedure have been reported (*1*). Glucuronidase activity was expressed as micrograms of phenolphthalein liberated per milligram of moist tissue after incubation for 1 hour with 0.0005 molar phenolphthalein glucuronide in acetate buffer at pH 4.5.

RESULTS AND DISCUSSION

The distributions of liver glucuronidase activity for the two parental strains, the F_1 and F_2 hybrid generations and the backcrosses to the strain C3H (C3HBC) and to the strain A (ABC) are shown in text-figure 1. The mean values and standard deviations are given in table 1. Sex did not appear to be a variable in liver glucuronidase determinations (*1*, *2*), nor



TEXT-FIGURE 1.—Distribution of liver glucuronidase activities in parental strains, C3H and A and in various crosses using C3H and A.

were any differences observed in the reciprocal crosses of the F_1 hybrid generation and in the backcross generations. Thus, the distributions in the text-figures and the values in table 1 represent combined sexes and combined reciprocal crosses, although the crosses are listed for simplicity as being accomplished in one direction only.

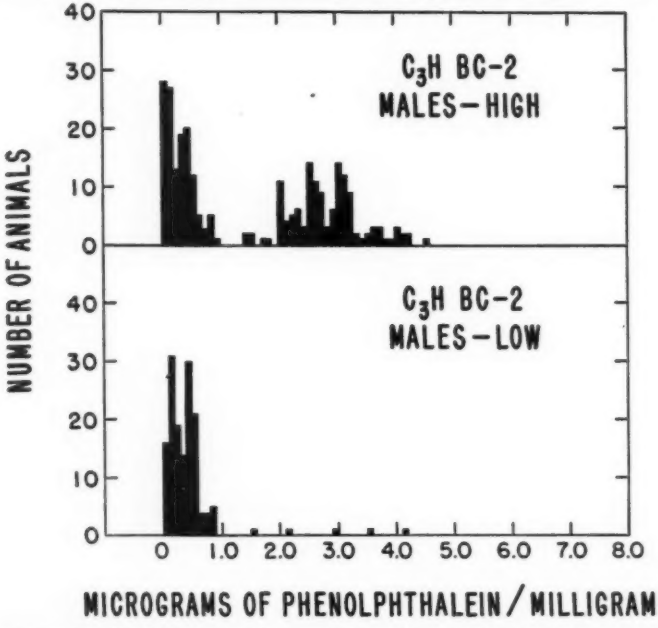
The mean glucuronidase activity of the F_1 hybrid generation closely approaches the value for the strain A (high activity), and the distribution of the values is similar. A definite bimodal distribution of glucuronidase activities in the two segregating generations, the F_2 and the backcross to

TABLE 1.—*Liver glucuronidase activity in strains, hybrids, and segregating generations in matings of C3H×A*

Strain or cross	Number of mice	Liver glucuronidase activity [1]		
		Mean		Standard deviation
		Low (<1.0 γ/mg.)	High (>1.0 γ/mg.)	
1 C3H×C3H	78	0. 25		0. 19
2 A×A	69		3. 73	. 68
3 F_1 (A×C3H)	192		2. 99	. 84
4 F_2 (A×C3H)×(A×C3H)	20	. 44		. 29
	80		2. 81	. 78
5 C3HBC (A×C3H)×C3H	134	. 36		. 22
	126		2. 77	. 82
6 ABC (A×C3H)×A	104		3. 02	1. 05
Second backcross:				
1 (C3HBC2) (high male)	132	. 29		. 16
	132		2. 88	. 62
2 (C3HBC2) (low male)	145	. 35		. 22

[1] Expressed as micrograms of phenolphthalein liberated per milligram of moist tissue after incubation for 1 hour with 0.0005 M phenolphthalein glucuronide in acetate buffer at pH 4.5.

the strain C3H (C3HBC), is evident, the modal class representing reduced glucuronidase activity being similar (mean value and standard deviation) to the C3H parental strain and the modal class representing high glucuronidase activity closely resembling the A parental strain. The back-



TEXT-FIGURE 2.—Distribution of liver glucuronidase activities in second backcross offspring (C3HBC×C3H) using *a*) backcross males with high glucuronidase activity, and *b*) males with low activity.

cross to the strain A (ABC) again resembles the values for the strain A although showing considerably more variability.

A bimodal distribution of values indicates the predominant action of one pair of genes influencing glucuronidase activity. Furthermore, the segregation ratios obtained, 20 low:80 high (1:3) in the F_2 generation and 134 low:126 high (1:1) in the C3HBC backcross generation are, within sampling errors, those expected assuming the differences in glucuronidase activity result from the actions of one pair of genes acting with dominance.

The distribution of liver glucuronidase levels for the second backcross generation (C3HBC2) using *1*) backcross males with low glucuronidase activity and *2*) those with high activity, is shown in text-figure 2. The means and variances for these crosses are given in table 1, and individual matings are listed in table 2.

This second backcross generation constitutes the critical test for genetic factors influencing liver glucuronidase levels, since it classifies each male, of a generation in which genetic segregation occurs, according to the glucuronidase levels of its offspring in a cross with the strain (C3H), showing low glucuronidase activity.

TABLE 2.—*Liver glucuronidase activity in offspring of a second backcross to the C3H strain using backcross males (C3HBC) individually characterized as to glucuronidase activity*

Backcross males		C3H females		Second backcross offspring			
Animal number	Glucuronidase activity	Number producing offspring	Mean glucuronidase activity	Number	Glucuronidase activity		
					Range	Number of mice (low: <1.0)	(high: >1.0)
(High glucuronidase activity)							
♂23	2. 6	1	0. 60	4	0. 42–3. 1	2	2
♂24	2. 4	2	. 53	19	. 03–3. 5	10	9
♂13	1. 8	5	. 64	36	. 05–4. 5	20	16
♂14	2. 2	3	. 54	27	. 04–4. 2	11	16
♂61	1. 8	6	. 43	48	. 06–3. 1	31	17
♂62	1. 9	6	. 33	71	. 20–4. 2	34	37
♂34	2. 2	2	(?)	10	. 20–3. 3	4	6
♂40	3. 9	4	(?)	44	. 02–3. 3	17	27
♂33	2. 4	1	(?)	5	. 14–3. 1	3	2
						132	132
(Low glucuronidase activity)							
♂51	0. 42	4	. 45	30	. 09– . 75	30	--------
♂36	. 28	4	. 26	34	. 11– . 85	34	--------
♂60	. 38	4	. 34	47	. 02–3. 4	42	5
♂43	. 46	1	(?)	4	. 43– . 49	4	--------
♂2	. 29	5	. 50	35	. 16– . 60	35	--------
						145	5

It can be seen from the figures and tables that the frequency distribution, mean, and variance of second backcross offspring produced by C3HBC backcross males [(A×C3H)×C3H] showing low glucuronidase activity

(mean for five males=0.37 γ/mg.) strikingly resembles the strain C3H. Second backcross offspring, resulting from crosses using backcross males with previously determined high glucuronidase activity (mean for nine males=2.36 γ/mg.) show a definite bimodal distribution of glucuronidase activity; the mean and variance of the low modal class being strikingly similar to the C3H (low) strain values and the mean and variance of the high modal class similar to the A (high) strain values.

It is apparent that the high activity of the enzyme, glucuronidase, in the liver of the mouse is dependent upon the presence of a single dominant gene, which we propose to designate *G*. The majority of the strains tested: A, C57 black, CBA, RIII, *etc.* (*2*) would thus be homozygous for this gene, *GG*. The strain C3H, which shows a low activity of liver glucuronidase would carry the recessive alleles, *gg*. The expected genotypes and phenotypes of the matings accomplished in this study would be:

Cross	Matings	Genotype	Expected phenotype	Observed phenotype
F_1	*GG* × *gg*	*Gg*	All high	192 high.
F_2	*Gg* × *Gg*	*GG, Gg, Gg, gg*	3 high: 1 low	80 high: 20 low.
C3HBC	*Gg* × *gg*	*Gg, gg*	1 high: 1 low	120 high: 134 low.
ABC	*Gg* × *GG*	*GG, Gg*	All high	104 high.
C3HBC2:				
High male	*Gg* × *gg*	*Gg, gg*	1 high: 1 low	132 high: 132 low.
Low male	*gg* × *gg*	*gg*	All low	5 high: 145 low.

It may be seen that the observed segregation ratios obtained in the various crosses closely fit the ratios expected of a trait determined by a single dominant gene. The slight deviation from a 3 : 1 ratio obtained in the F_2 population is clearly the result of sampling: $\chi^2=1.08$; $P=0.33$. The 5 exceptions that appeared among 150 offspring of the 2 BC mating are difficult to explain. These mice, having glucuronidase activity values of 1.6, 2.2, 2.9, 3.4, and 4.1 γ/mg. resulted from a cross of $BC_1♂$ #60, having a glucuronidase activity of 0.38 γ/mg. with 3 different strain C3H females whose glucuronidase activity levels were 0.38, 0.43, and 0.27 γ/mg., respectively. It is clear that these exceptional individuals, exhibiting high glucuronidase levels, which appeared in a cross between animals showing low glucuronidase activity, are not deviations from expected ratios and probably require a genetic mechanism other than the primary gene involved determining glucuronidase activity, since they have not been observed in the inbred strain C3H showing low activity. Similar evidence for the occurrence of genetic variables, aside from the primary gene, has been reported by MacDowell and Richter (*4*) in crosses between inbred strains of mice susceptible to, and resistant to, a transplantable leukemia.

Segregation for three coat color genes, brown (*Bb*), Agouti (*Aa*) and albinism (*Cc*) occurred in the F_2 and ABC backcross generations. Analysis of the data indicates no specific association between these genes and glucuronidase activity.

The present data give evidence of another example of the relationship between gene and enzyme. It appears that as a result of a defective hereditary factor, or gene, a specific enzyme, glucuronidase, is likewise defective, the situation resembling closely the classic example of hereditary alcaptonuria in man (*5*).

There appears to be no established relationship between glucuronidase activity and incidence of spontaneous tumors in mice (*2*). It is quite clear, however, that in man (*6,7*) high glucuronidase activity is associated with a number of cancerous tissues, and in mice mammary gland tumors of several different inbred strains are associated with increased glucuronidase levels (*8*). Experiments are now in progress investigating the role of glucuronidase in the production of spontaneous hepatomas in mice.[6]

SUMMARY

Matings were made between two strains of mice, C3H and A, differing in liver glucuronidase activity. The strain C3H consists of mice exhibiting a low glucuronidase activity, 0.25 γ/mg. per moist weight of liver tissue. The A strain consists of mice with a high glucuronidase activity, 3.73 γ/mg.

The F_1 and A backcross generations gave distributions closely resembling the A parental strain, whereas the F_2 and C3H backcross generations gave distinct bimodal distributions, the lower modal class in each case being strikingly similar to strain C3H and the higher modal class similar to strain A.

C3H backcross males with either high or low liver glucuronidase activity, as determined by partial hepatectomy, were mated to strain C3H females to produce second backcross offspring. The distributions and ratios obtained were those expected of segregation of a single gene-pair showing a dominance-recessive relationship.

Mean glucuronidase activity was similar in reciprocal crosses, indicating absence of any maternal influence.

There was found to be no association between glucuronidase activity and any of three coat color genes: *Aa*, *Bb*, and *Cc*.

The symbol *g* is suggested to designate the recessive allele indicating low liver glucuronidase activity.

No established relationship between glucuronidase activity and spontaneous tumor incidence is apparent.

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ACID AND ALKALINE PHOSPHATASE OF SERUM AND HEPATIC TISSUE OF RATS FED CARCINOGENS [1]

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It has been shown that tumors of the liver in the rat have a higher alkaline phosphatase concentration than normal liver. Greenstein (*7*), working with transplanted Hepatoma 31, found that alkaline phosphatase in this tumor tissue was about 200 times as high as that of normal rat liver. Woodard (*20*) reported a tenfold increase in alkaline phosphatase values for tumor tissue in albino rats fed a diet containing *p*-dimethylaminoazobenzene, in comparison with alkaline phosphatase values of normal liver.

The present study is an attempt to determine whether the changes in the acid and alkaline phosphatase values precede or follow the development of tumors in the liver, and whether such changes may be correlated with histologic changes in the liver during development of a neoplasm.

MATERIAL AND METHODS

Two hundred and five male Osborne-Mendel rats about 3 months old, 150–200 gm. in weight, were fed a basic semisynthetic, low-protein diet consisting of 78 percent cerulose-sugar, 12 percent low-vitamin casein, 4 percent salt mixture, 1 percent vitamin mixture, and 5 percent corn oil. Sufficient carcinogen was incorporated in the corn-oil fraction to bring the final concentration of the carcinogen in the diet to 0.075 percent for 4-dimethylaminobenzene-1-azo-1-naphthalene (DAN), or 0.06 percent for *p*-dimethylaminoazobenzene (DAB). One group of 80 animals was kept on the diet containing DAN; 20 of these rats for a period of 230 days, and the remaining 60 for 280 days. A second group of 85 animals was kept on the diet containing DAB; 20 rats for a period of 150 days, and 65 for 280 days. A third group of 40 control rats was kept for 280 days on the basic semisynthetic diet without any carcinogen.

Rats in all three groups were returned to a diet consisting of Purina laboratory chow pellets at the end of the stated period on the experimental diets.

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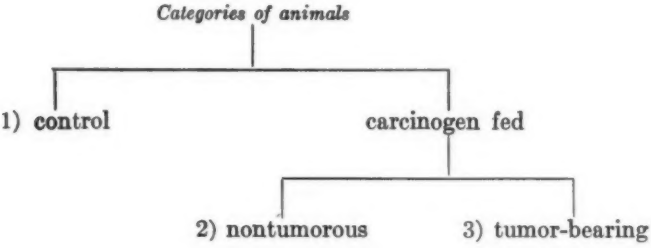
Animals were sacrificed at various intervals. The first rat was killed at 2 months and the last one at 25 months after the experiment began. Control animals were sacrificed at about the same time as the ones receiving the carcinogens. The age of the rats at the time of sacrifice ranged from 5 to 28 months.

For the purposes of the serum phosphatase studies, the animals were divided into three groups: 1) *Control* rats on the basic diet without carcinogen; 2) *Nontumorous* rats receiving one or other of the carcinogenic dyes sacrificed before they developed liver tumors; and 3) *Tumor-bearing* rats receiving one or other of the carcinogenic dyes, but sacrificed after they developed liver tumors.



Blood for phosphatase study was collected by cardiac puncture from the rats anesthetized with ether just before autopsy. The serum was separated from the blood and stored in the icebox until tested.

All livers were carefully examined for tumors with a magnifying glass at the time of autopsy. As a rule, a single piece of liver tissue was sufficient for a one-gram sample, but in a few cases, pieces from different lobes of the same liver were pooled to make up the sample. A single piece from one tumor was always sufficient for a one-gram sample. A piece of tissue adjacent to the sample removed for phosphatase determination, and similar in gross appearance to the phosphatase sample, was removed in every case for microscopic examination. Specimens for histologic study were fixed in Zenker-acetic fluid, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Gomori's methods for acid (*6*) and alkaline (*5*) phosphatase were used for histochemical demonstration of these enzymes.

Each sample of liver taken for phosphatase determination was placed in one of the following categories, depending upon the type of hepatic tissue and on the animal in which it was found:

(*a*) *Control liver.*—Liver tissue of animals that did not receive the carcinogens in their diet. In all other details except carcinogen, these animals received the same treatment as carcinogen-fed animals.

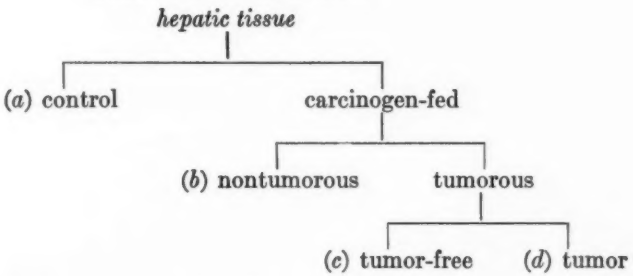
(*b*) *Nontumorous tissue of nontumor-bearing liver.*—Liver tissue of carcinogen-fed animals that showed neither gross nor microscopic evidence of tumor tissue anywhere in the liver.

(*c*) *Tumor-free tissue of tumor-bearing liver.*—Liver tissue of carcinogen-fed animals taken from areas free of tumor as far as could be determined

by gross examination with the aid of a magnifying glass. However, all these livers showed either gross or microscopic evidence of tumor of the liver in other areas. It is realized that these samples may possibly have possessed microscopic foci of tumorous tissue.

(*d*) *Tumor tissue of tumor-bearing liver.*—Neoplastic tissue from carcinogen-fed animals selected by gross examination and the diagnosis verified by microscopic examination of the adjacent pieces from the tumor removed for the purpose. All of the adjacent uninvolved tissue was trimmed from tumor tissue with the aid of a magnifying glass. These samples may possibly have had some microscopically identifiable uninvolved liver tissue included in them.

Categories of hepatic tissue



At first, the liver sample was assigned to one of these categories after gross examination with the aid of a magnifying glass. If subsequent microscopic diagnosis proved to be at variance with the gross observation, a new assignment was made to conform to the microscopic diagnosis. Every diagnosis of hepatic tumor made grossly at autopsy was confirmed by subsequent microscopic examination. Microscopic examination revealed approximately 7 percent of all cases to possess additional minute tumors of the liver that escaped detection at gross autopsy.

Samples of liver and tumor tissue taken for the phosphatase analysis were cut into cubes about 5 by 5 by 5 mm., and stored at once in covered petri dishes in the deep freeze until the chemical determinations were to be made. All phosphatase determinations were finished within 6 hours after the animal was killed. About one gram of tissue, the exact weight of which had been determined, was ground into paste in a mortar with sand and water. This paste was suspended in about 20 ml. of water and, after heavy particles settled out, the supernatant suspension was made up to 25 ml.

Alkaline and acid phosphatase determinations were made by modification of the King and Armstrong (*11*) and Gutman and Gutman (*10*) methods. Sodium acetate-acetic acid buffer was used in place of the sodium citrate-citric acid buffer used by Gutman and Gutman in the acid phosphatase determinations. Substrate and buffers were prepared separately and stored in the icebox. Their concentrations were so arranged that when one volume of buffer was added to one volume of substrate, the resulting mixture was of the desired pH and substrate concentration.

Buffered substrate was prepared, just before use, and its pH electrometrically checked.

Aliquots of either 0.1 or 0.2 ml. of the sample to be examined were incubated with 4 ml. buffer substrate in 100 × 13 mm. test tubes. Then enough dilute phenol reagent was added to each tube to bring its final volume to 6 ml. A 2-ml. sample of the filtrate was incubated with 0.5 ml. of 20 percent sodium carbonate for 5 minutes at 37.5° C. for color development. Colors were read on a Coleman Junior spectrophotometer at 540 mμ and the results calculated from a standard phenol curve prepared for the purpose.

RESULTS

The two carcinogens induced similar changes in the phosphatase values in relation to histopathologic changes and tumor development; therefore, the results were grouped together.

Detailed description of the histopathologic changes in the liver of carcinogen-fed rats is being published separately (*4,14*). It may be stated here, however, that in animals fed *p*-dimethylaminoazobenzene (DAB), the earliest tumors developed after 150 days, while in those fed 4-dimethylaminobenzene-1-azo-1-naphthalene (DAN), the first tumors appeared at 335 days. Liver tumors continued to appear for 9 months after the initial induction date in the case of DAB, and for 14 months in case of DAN. Thus, the age of the animal for which phosphatases were determined ranged from 5 to 28 months. Phosphatases were determined for control rats with a similar range of age. No correlation was discovered between age and phosphatase values.

The liver of both groups of carcinogen-fed rats began to show proliferation of bile ducts as early as 2 months following the beginning of the administration of carcinogen in the diet. Subsequently, there was a progressive increase in the number and size of the areas of bile-duct proliferation. These proliferative changes in the bile ducts were accompanied by minimal but increasing degrees of fibrosis. In some areas, there was atrophy of bile ducts. In many areas, scattered inflammatory cells and cellular debris in the bile ducts were found. The liver of the animals that showed bile-duct proliferation also showed dilatation of the ducts and formation of cystic structures that were often multiloculated and filled with clear yellow or green fluid. Concomitant with these changes in the bile ducts, degeneration of hepatic cells in the central zones of the lobules occurred. Subsequently, the hepatic parenchymal cells regenerated in the periphery of the lobules. Still later in the course of feeding carcinogen, nodules of newly formed cells that had lost normal orientation, no longer forming liver cords, were seen. After initiation of the administration of DAB and DAN, about 6 and 11 months, respectively, tumors began appearing in single or multiple areas throughout different lobes of the liver. These tumors were divided into three groups: A) hepatoma; B) adenocarcinoma (cholangioma); and C) cholangiohepatoma, a mixture of types

A and B. The hepatoma, in general, was a solid mass of proliferating hepatic cells with focal areas of necrosis and sometimes hemorrhage. The cholangiomas, as will be described in a separate publication (*4*), frequently contained areas of mucin-like material and moderate amounts of fibrous stroma. The third group, cholangiohepatoma, consisted of an intermixture of these two morphologic types of tumor.

The mean values and ranges of acid and alkaline phosphatase of the sera of carcinogen-fed and normal animals and the percentage distribution of these ranges in the population are shown in tables 1 and 2. The acid and alkaline phosphatase values for liver and tumor tissue are given in tables 3 and 4 and correlated with the type of tissue analyzed.

TABLE 1.—*Serum acid phosphatase in rats with and without induced liver tumors*

Category of animals	Number of animals studied	Mean value in units [1]	Range in units [1]	Percentage of animals having enzyme activities in units [1] of:				Over 12 units
				3	3. 1–6	6. 1–9	9. 1–12	
Tumor-bearing [2]	40	7. 5	1. 2–16. 6	7	33	37	16	7
Nontumorous [3]	20	8. 0	3. 6–16. 3	------	40	20	30	10
Control	30	7. 4	3. 1–17. 8	------	26	53	13	7

[1] Units per 100 ml. serum.
[2] Carcinogen-fed rats with tumors of the liver.
[3] Carcinogen-fed rats with no tumors of the liver.

TABLE 2.—*Serum alkaline phosphatase in rats with and without induced liver tumors*

Category of animals	Number of animals studied	Mean value in units [1]	Range in units [1]	Percentage of animals having enzyme activities in units [1] of:						Over 70 units
				11–20	21–30	31–40	41–50	51–60	61–70	
Tumor-bearing [2]	40	48	12–84	2	13	22	13	22	22	6
Nontumorous [3]	20	28	18–38	20	40	40	----	----	----	----
Control	30	32	11–60	27	40	10	7	16	----	----

[1] Units per 100 ml. serum.
[2] Carcinogen-fed rats with tumors of the liver.
[3] Carcinogen-fed rats with no tumors of the liver.

SERUM ACID PHOSPHATASE

The different groups of carcinogen-fed and control rats did not show significant differences in their values of the serum acid phosphatase (table 1). This indicates no relationship of acid phosphatase activity to the morphology of the liver lesions. The mean phosphatase value, the spread of the range, and the percentage distribution in the range were similar in control, nontumorous, and tumor-bearing animals. The range for tumor-bearing animals began at a little lower point than did that of the other two groups. However, only about 7 percent of the rats showed phosphatase values in this lower range.

TABLE 3.—*Acid phosphatase in hepatic tissue of rats with and without induced liver tumors*

Category of hepatic tissue	Number of animals studied	Mean value in units [2]	Range in units [1]	Percentage of animals having enzyme activities in units [1] of—						Over 3,000 units
				500	501–1,000	1,001–1,500	1,501–2,000	2,001–2,500	2,501–3,000	
Hepatic tumor	50	1, 073	53–3, 230	26	24	24	18	6	----	4
Tumor-free [2]	50	1, 800	290–2, 920	10	----	14	40	20	16	----
Nontumorous [3]	23	1, 970	860–3, 500	----	9	13	47	22	----	9
Control	28	1, 830	970–2, 840	----	7	14	46	26	7	----

[1] Units per 100 gm. fresh tissue.
[2] Liver tissue free of tumors from tumor-bearing liver.
[3] Liver tissue from carcinogen-fed rats, but without liver tumors.

TABLE 4.—*Alkaline phosphatase in hepatic tissue of rats with and without induced liver tumors*

Category of hepatic tissue	Number of animals studied	Mean value in units [2]	Range in units [1]	Percentage of animals having enzyme activities in units [1] of—								Over 1,000 units
				25	25–50	51–100	101–150	151–200	201–250	251–500	501–1,000	
Hepatic tumor	50	496	109–1, 190	----	----	----	10	8	10	26	42	4
Tumor-free [2]	50	109	13–226	4	8	38	30	12	8	----	----	----
Nontumorous [3]	23	93	21–173	14	14	29	29	14	----	----	----	----
Control	28	49	17–78	22	22	56	----	----	----	----	----	----

[1] Units per 100 gm. fresh tissue.
[2] Liver tissue free of tumors from tumor-bearing liver.
[3] Liver tissue from carcinogen-fed rats but without liver tumors.

Serum Alkaline Phosphatase

The serum alkaline phosphatase (table 2) in the tumor-bearing rats showed a significant rise over that of the nontumorous (carcinogen-fed) and the control animals. The mean alkaline phosphatase value of the tumor-bearing rats had shifted upward by about 50 percent as compared with the mean value of the control rats, and the range was extended upward by 24 units. In the control animals, about three-fourths of the values fell below the mean value of 32; while in tumor-bearing animals almost three-fourths of the values were above this mean value and about one-third of the values were above the highest alkaline phosphatase value of the controls. Bernard and Rosenbloom (*1*) reported a similar association of high serum alkaline phosphatase values with cancer in man. These authors stated that this is especially true for primary cancer of the liver. A study of the distribution of the values for serum alkaline phosphatase among nontumorous (carcinogen-fed) rats revealed no significant change from that of the control animals.

Acid Phosphatase in Hepatic Tissues

Analysis of the acid phosphatase activity of the hepatic tissue of normal and carcinogen-fed rats (table 3) revealed a significant shift toward lower values for tumor tissue, while no significant change from the normal distribution was observed in either the nontumorous (liver of carcinogen-fed rats without tumors) or the tumor-free (tumor-free liver from tumor-bearing rats) liver tissue. Half of the acid phosphatase values for tumors of the liver were under 1,000, while only about a tenth of the other types (nontumorous and tumor-free) of liver tissue examined fell in this low range. The mean value of acid phosphatase activity of tumors of the liver was almost half that of the control liver tissue. The mean acid phosphatase values of control, nontumorous, and tumor-free hepatic tissues revealed no correlation with histologic structure. However, low acid phosphatase values were found in tumorous tissue.

Mean value of acid phosphatase activity of the liver-cell tumor tissue was about 70 percent higher (1,297 units) than that of cholangiohepatoma tissue (765 units). There were no values under 500 units in the former group, while almost half the values in the latter group were under 500 units, 170 units being the lowest value. The highest acid phosphatase value of liver-cell tumor tissue was 3,200 units, while that of cholangiohepatoma tissue was 1,750 units.

Alkaline Phosphatase in Hepatic Tissues

The alkaline phosphatase of hepatic tissues showed progressively higher mean values and wider ranges from the control, through nontumorous (liver of carcinogen-fed rats that showed no evidence of tumor), tumor-free (liver of tumor-bearing rats, taken from areas free of tumor), to actual tumor tissue (table 4). The range of alkaline phosphatase values for tumor tissue started where that of control values ended, and the mean of the former was 10 times as high as that of the latter. The nontumorous

and the tumor-free liver tissues had ranges that covered the control zone and extended into the zone of values for hepatic tumor tissue. The tumor-free hepatic tissue had fewer values in the range below 50 than did the nontumorous hepatic tissue. The mean for the tumor-free liver tissue was higher than that for the nontumorous tissue, and both were about twice as high as that of the control liver tissue. This progressive increase in alkaline phosphatase activity in the liver, in carcinogen-fed rats shows a positive correlation with the degree of pathologic change in the liver tissue, since many liver-phosphatase values for control and carcinogen-fed animals lie in the 51–100 unit range (table 4). This range was divided into two parts: a) 51–75 units, and b) 76–100 units. Fifty-three percent of the control values fall in the 51–75 unit range, while 30 percent of the tumor-free and 25 percent of the nontumorous values fall in the 76–100 unit group. Thus, about 65 percent of alkaline phosphatase values for the nontumorous liver tissue and 75 percent of those for the tumor-free liver tissue were found to be above the range of values for the control liver tissue.

The mean value for alkaline phosphatase of the liver-cell tumor tissue was slightly higher (546 units) than that of cholangiohepatoma tumor tissue (408 units). The lowest value for alkaline phosphatase found in either group was 150 units. The highest value for alkaline phosphatase in cholangiohepatoma tissue was 800, while there were many cases of liver-cell tumor tissue having much higher values (highest value 1,190 units).

DISCUSSION

The acid phosphatase activity of neither the serum nor the liver tissue showed any relation to the progressive pathologic changes in the liver brought about by feeding carcinogens to rats. However, the acid phosphatase activity of actual tumor tissue itself is low. Hence, these determinations of acid phosphatase have little diagnostic value for cancer of the liver. In 30 percent of the cases high alkaline phosphatase content of serum was correlated with the presence of tumors of the liver. Since 70 percent of the tumors in rats could well be missed by this criterion, alkaline phosphatase determination of serum would have very limited diagnostic value for cancer of the liver.

On the other hand, the amount of alkaline phosphatase present in hepatic tissue is definitely correlated with the course of pathologic change from normal to neoplastic hepatic tissue. Although this correlation is by no means absolute, 65 percent of the nontumorous and 75 percent of the tumor-bearing animals may be distinguished from the control rats by the higher alkaline phosphatase content of their liver tissue. Thus, the alkaline phosphatase determination under the conditions of this study detected three-fourths of the rats bearing tumor of the liver and two-thirds of the cases in which liver tumors may have developed at some time, if they had been allowed to live long enough. The assumption of the probable development of tumors in these rats seems justified, since about

90 percent of the rats fed these carcinogenic azo dyes eventually develop hepatic tumors.

The present study suggests a progressively greater increase in proliferation of bile ducts and of hepatic-cell tissue and ultimate appearance of neoplasms in many cases of Osborne-Mendel rats fed azo dyes (*p*-dimethylaminoazobenzene or 4-dimethylaminobenzene-1-azo-1-napthalene). These changes are described in detail elsewhere (*4, 14*). This confirms the findings of Pearson and Marrione (*17*), who described such proliferation of tissues preceding the formation of hepatomas in Sherman strain rats fed *p*-dimethylaminoazobenzene. These authors also found that alkaline phosphatase activity of the liver tissue, as determined by histochemical methods, was proportional to the degree of their resemblance to small proliferating bile ducts. Woodard (*20*), using chemical methods, found a higher mean value and a wider range of values of the liver alkaline phosphatase in rats fed a carcinogenic diet than those of the livers of normal rats. However, Greenstein (*8, 9*) found no increase in alkaline phosphatase activity either in regenerating liver or in livers of rats bearing tumors.

The proliferation of bile ducts and hepatic cells in response to the carcinogenic agent appears to be associated with a rise in liver alkaline phosphatase. This rise was previously shown to occur also during regeneration following partial hepatectomy, for Norberg (*15*) reported increase in the liver alkaline phosphatase (as high as 700 percent over that of normal liver) of regenerating rat liver after excision of a portion of the organ. Oppenheimer (*16*) found an increase in alkaline phosphatase in both the liver and the plasma of rats during regeneration of liver tissue. Sulkin (*19*) observed alkaline phosphatase activity histochemically in regenerating liver tissue but no activity in normal rat liver. Cleveland and his associates (*3*) concluded from their histochemical studies that the distribution of alkaline phosphatase in liver tissue had no value in the diagnosis of hepatoma in man.

A close association between alkaline phosphatase and ribose nucleic acid in macromolecular particles was shown by the fractionation studies of Brachet and Jenner (*2*), Steinbach and Moog (*18*) and Moog and Steinbach (*13*). Increase in the alkaline phosphatase content of hepatic cells is believed to be associated with resynthesis of nucleic acid and thus with increased nuclear activity (*12*). Increased liver alkaline phosphatase activity in regenerating liver reported by other workers lends support to the correlation between the alkaline phosphatase content of the liver and the degree of its proliferative activity as suggested by this study. Association of alkaline phosphatase with ribose nucleic acid by study of macromolecular granules (*2, 13, 18*) and the relation of this enzyme to nuclear activity (*12*) would seem to throw some light on the close relationship between liver alkaline phosphatase and proliferative activity.

These observations suggest that further study of phosphatase of the liver may lead to information that may be helpful in the differential diagnosis of cancer of the liver. Since the technique of securing a biopsy

specimen of the liver is a comparatively new diagnostic tool, very little data are available on changes in alkaline phosphatase of the liver of man in health and disease. When this information becomes available, more intelligent use could be made of the alkaline phosphatase test as an adjunct to diagnosis of malignancy.

SUMMARY AND CONCLUSIONS

The acid and alkaline phosphatase values of serum, liver tissue, and hepatoma were determined chemically in rats fed carcinogens (*p*-dimethylaminoazobenzene or 4-dimethylaminobenzene-1-azo-1-naphthalene). There was no appreciable difference between acid phosphatase content of the blood serum of control, nontumorous, and tumor-bearing rats. A rise in the serum alkaline phosphatase was demonstrated in 28 percent of the animals bearing tumors of the liver.

Acid phosphatase values for hepatic tumor tissue had much wider range and lower mean value than those of normal liver tissue of control rats. Fifty percent of the values for hepatic tumors were below the range of values for normal liver.

Lowest alkaline phosphatase values of hepatic tumors were higher than the highest value of normal liver and the highest values were as high as 24 times the mean value of normal liver. The mean value of hepatic tumors was 10 times greater than that of normal livers. Both acid and alkaline phosphatase values for liver-cell type tumor tissue were higher than those for cholangiohepatoma type tissue.

The mean alkaline phosphatase value for liver of carcinogen-fed rats was twofold greater than that of the liver of the control animals. Sixty-five percent of the nontumorous liver of carcinogen-fed rats and 75 percent of the tumor free tissue of livers bearing tumors showed alkaline phosphatase values that were above the range of normal liver values.

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METABOLISM OF L-HISTIDINE-2-C^{14} IN THE NORMAL AND THE HEPATOMA-BEARING RAT [1]

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INTRODUCTION

Comparatively little information of an unequivocal character is available concerning the intermediary metabolism of histidine in mammals. One early postulate suggested by the presence of an imidazole ring in the histidine molecule is that histidine is a precursor of purines (*1*). The concept that the imidazole ring is used as a nucleus upon which the remainder of a purine structure is built has been excluded, however, by Tesar and Rittenberg (*2*), who showed by tracer technique that the γ-nitrogen of the ring is not selectively utilized for the formation of urinary allantoin or liver purines in the rat, and Broquist and Snell (*3*) have concluded from an analysis of growth requirements of several microorganisms that histidine does not function as a purine precursor. Nevertheless there remains a substantial if somewhat conflicting literature on feeding and perfusion experiments, which contains numerous reports of increased purine formation following histidine administration (*4–10*).

Another possibility originally suggested by the presence of the imidazole nucleus is that opening of the ring might lead to a guanidine nucleus which would be used in the synthesis of creatine (*1*), and the preponderance of data obtained *in vitro* and from feeding experiments indicates that histidine does serve as a creatine precursor (*4–6*, *11*, *12*). However, failure of the γ-nitrogen of the ring to be selectively utilized for this purpose (*2*) suggests that an indirect mechanism is involved.

The known behavior of histidine in releasing the carbon atom at position 2 of the ring as formate under conditions of acylative hydrolysis (*13*) suggests that histidine functions as a biochemical formate source. This supposition is strengthened by the existence of the enzyme histidase, capable of transforming histidine to a substance that is hydrolyzed to formate on treatment with boiling mineral acids or alkali (*14*). Formate is a source of labile methyl groups (*15*), which in turn are used for the synthesis of creatine (*16*), and formate is also a purine precursor (*17*). Therefore, if histidine is biologically converted to formate the ambiguities

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noted above can be somewhat clarified. In this paper, which extends a preliminary communication (*18*), data are presented showing that this conversion does occur.

The subject of purine metabolism occupies an important position in the biochemistry of cancer for well-known reasons. Formate metabolism, too, assumes considerable interest when recent observations implicating folic acid in formate and methyl group metabolism (*19, 20*) are considered together with the effect of certain folic acid analogues as cancer inhibitors (*21*). For these reasons there has been included in the study an examination of this histidine-formate relationship in cancer-bearing rats.

EXPERIMENTAL PROCEDURE

Synthesis of labeled histidine.—Subsequent to our preliminary account of the histidine synthesis (*22*), there appeared a report by Borsook *et al.* (*23*) which also describes the synthesis of histidine-2-C^{14}. The methods used by the two groups of workers are essentially identical and we therefore record only a few notes on the procedure.

Sodium thiocyanate-C^{14} was prepared by refluxing commercial sodium cyanide-C^{14} for 1 hour with a 20-percent excess of sulfur in ethanol. Free alkali present in the cyanide caused difficulty by giving rise to complex sulfur compounds. For this reason there was added to the initial mixture a quantity of ethyl carbonate equivalent to that of the alkali. The carbonate hydrolyzed rapidly and by reducing the basicity of the medium reduced the extent of the side reaction. After precipitation of dissolved sodium carbonate by saturation of the solution with carbon dioxide a clean preparation of sodium thiocyanate was obtained in quantitative yield by evaporation of the alcohol.

Radiochemical purity of the labeled histidine was checked by paper chromatography in phenol and in lutidine. Autoradiography of the chromatograms showed only one radioactive spot.

The possibility of partial racemization of the alpha position was checked by mixing a small quantity of the radioactive preparation with an authentic specimen of D-histidine and recrystallizing the mixture to constant specific activity. The final specific activity was 1.3 percent of the original, and the concentration of D-histidine in the preparation is therefore not greater than this figure.

The specific activity of the labeled histidine, which was prepared from 5 mc. of sodium radiocyanide, was 1.1 mc. per mM.; the over-all yield was 7.1 percent.

Metabolic experiments.—Adult Osborne-Mendel rats in two groups of three were each given by stomach tube 2.33 mg. of labeled histidine. The dose was 3.7×10^7 disintegrations per minute. The rats in one group were normal; the others bore primary hepatomas induced by feeding *p*-dimethylaminoazobenzene. Histologic examination established the tumors as malignant neoplasms of mesenchymal origin.[3] During the

[3] We thank Dr. Thelma B. Dunn for the pathologic examinations.

experimental period the animals were fed *ad libitum* a diet [4] composed of 60 percent sucrose, 19 percent Crisco, 12 percent casein (crude), 4 percent modified salt mixture (*24*), and 5 percent corn oil (Mazola). To each kilogram of diet was added the following supplement: 3 mg. thiamine chloride, 2 mg. riboflavin, 2.5 mg. pyridoxine hydrochloride, 7 mg. calcium pantothenate, 30 mg. choline chloride, and 100 mg. nicotinic acid hydrochloride. This diet supports normal growth, but is not overly rich in protein or methyl groups.

Urine was collected and analyses were made on aliquots; allantoin was determined by the method of Young and Conway (*25*); uric acid by the uricase method (*26*); creatine and creatinine by the method of Folin, and urea by incubation with urease. Carbon dioxide formed from the urea was collected in barium hydroxide, and the barium carbonate was used for radioactivity assay. It was desired to isolate substantially all of each of these metabolites (except urea) rather than to isolate them individually from aliquots. In order to accomplish this without encountering cross interferences between various operations the following procedure was adopted: To 30.0 ml. (nearly all) of a diluted urine specimen were added as carriers 25 mg. of uric acid dissolved in 0.6 ml. of 0.5 M sodium hydroxide, 50 mg. of allantoin, 25 mg. of creatine, and 25 mg. of creatinine; all accurately weighed. These substances were then isolated in the following sequence:

Uric acid.—To the urine was added hydrochloric acid to a final concentration of 0.1 M, and the solution was allowed to stand 3 days at 5° C. The precipitate of crude uric acid was dissolved in 0.5 ml. of 0.5 M sodium hydroxide, treated with Norite, and recovered by acidification. It was reprecipitated until constant specific activity was attained. As an independent purification procedure the purified uric acid was dissolved in concentrated sulfuric acid and allowed to stand 1 hour. The solution was nearly colorless. The specific activity of the uric acid, recovered by dilution with water was raised slightly by this treatment.

Allantoin.—To the filtrate obtained after removal of uric acid was added 10 ml. of 5 percent mercuric chloride, and the solution was allowed to stand overnight. A slight precipitate was removed. The solution was nearly neutralized with sodium carbonate and was adjusted to pH 8 with sodium bicarbonate. The mercury complexes of allantoin and creatinine that precipitated were washed with water and extracted with two 20-ml. portions of 0.1 M hydrochloric acid. After removal of undissolved material the complexes were reprecipitated from the filtrate and the procedure was repeated until a product completely soluble in tenth molar hydrochloric acid was obtained. Mercury was then removed with hydrogen sulfide, and the solution was lyophilized. The allantoin was purified by recrystallization from water to constant specific activity.

Degradation of the allantoin was accomplished by alkaline hydrolysis to allantoic acid followed by hydrolysis to glyoxylic acid and urea with hydrochloric acid (*25*). The acid hydrolysis was carried out in the

[4] The diet is a formulation used by Dr. Julius White. We wish to thank him for supplying the diet and for the tumor-bearing rats.

presence of 2,4-dinitrophenylhydrazine. The glyoxylic acid was isolated as the dinitrophenylhydrazone; urea was isolated as the xanthydrol derivative.

Creatine and creatinine.—To isolate creatinine, the first supernatant solution from recrystallization of the allantoin was adjusted to pH 10 with ammonia, and the solution was passed through a 1 by 10 cm. column of Dowex-1 anion exchanger resin, which removed most of the radioactive impurities. The creatinine was isolated from the effluate as the zinc chloride complex, which was recrystallized from dilute ethanol to constant specific activity.

The urine solution remaining after removal of allantoin and creatinine was made 1 M in hydrochloric acid and boiled 1.5 hours to convert creatine to creatinine, which was isolated and purified as before.

Confirmatory evidence for the radiochemical purity of the allantoin and creatinine was obtained by chromatography. Allantoin was placed on a 1 by 40-cm. column of potato starch (Eimer and Amend C. P. grade) as a saturated solution in a solvent consisting of formic acid, water and *t*-butanol in the proportions 15:15:70. The allantoin was eluted with the same solvent at a flow rate of 1.5 ml. per hour and collected in 1-ml. cuts. The recovery in this system is quantitative. Creatinine-zinc chloride was placed on a 30 by 1-cm. column of Dowex-50 cation exchange resin and eluted with 0.1 percent ammonia. The zinc remained on the column. Chromatography did not change the specific activities, and a plot of total compound eluted against total radioactivity eluted was linear. The chromatographic criterion was not employed routinely after it was demonstrated that the isolation methods give radiochemically pure products.

Choline and cholesterol.—Lipids were extracted from vacuum-dried liver tissue with hot 3:1 alcohol-ether, and the extract was hydrolyzed with 100 ml. of saturated barium hydroxide solution. The organic solvent was allowed to boil off, and the mixture was cooled and filtered. Cholesterol was isolated from the residue as the digitonide; choline was isolated from the filtrate as the reineckate (*27*).

The cholesterol was recovered from the digitonide by treatment with pyridine and ether (*28*) and recrystallized from dilute ethanol. The choline reineckate was purified by conversion to the chlorplatinate.

Degradation of the choline chlorplatinate was effected with alkaline permanganate (*16*).

RESULTS

In table 1 are listed the radioactivity assay data. The values are corrected for isotopic dilution in the cases where that isolation technique was employed.

Normal group.—Uric acid and allantoin, the purine and products, acquire significant radioactivity promptly after histidine administration. Comparison of these values with those for urea, which represent the average radioactivity level of the respiratory carbon dioxide during the experimental period (*29*), shows that most of the radioactivity in the purines arises by processes other than carbon-dioxide fixation. Upon

degradation of the 72-hour specimen of allantoin to urea and glyoxylic acid, all of the radioactivity was found in the urea within an experimental error of 9 percent. Confirmatory examination of the glyoxylic acid failed to reveal any detectable radioactivity. Since in the biologic conversion of uric acid to allantoin the ureido carbons are derived from positions 2 and 8 of uric acid, the results of the allantoin degradation are in accord with the statement of Sprinson (*30*) that histidine contributes carbon to positions 2 and 8 of uric acid.

TABLE 1.—*Radioactivity of the metabolites* [1]

	Normal			Hepatoma-bearing		
	12 hours	24 hours	72 hours	12 hours	24 hours	72 hours
Uric acid	44. 0	27. 5	29. 4	43. 8	63. 2	31. 6
Allantoin	59. 5	49. 0	49. 2	84. 9	------	43. 6
Creatine	3. 78	1. 52	------	------	------	------
Creatinine	------	------	. 40	------	------	------
Urea	4. 03	5. 06	2. 61	1. 25	1. 53	. 613
Erythrocyte protoporphyrin	0	------	0	0	------	0
Cholesterol:						
Normal liver	5. 56	------	------	------	------	------
"Precancerous" liver [2]	------	------	------	------	------	1. 3
Hepatoma	------	------	------	------	------	1. 2
Choline:						
Normal liver	44. 2	47. 3	50. 0	------	------	------
"Precancerous" liver	------	------	------	33. 9	44. 5	61. 1
Hepatoma	------	------	------	8. 66	19. 0	54. 8

[1] Data are expressed as specific activity (dpm/μ mole) $\times \frac{\text{Body weight (gm.)}}{100} \times \frac{1}{\text{Dose (microcuries)}}$.

[2] By precancerous liver is meant the liver tissue that remains after removal of the tumor.

The creatine and creatinine values lie below those of urea and since it is known that carbon dioxide is actively fixed in these compounds (*31*), a substantial portion of the radioactivity may arise from this source. Actually, as is shown below, histidine is a precursor of choline methyl groups and part of the creatinine activity must therefore be in its methyl groups (*16*). The values for creatine and creatinine, which may be regarded as end products of methyl group metabolism, show that the relative contribution of histidine to the animal's total purine supply is much greater than to its methyl group supply.

These results reconcile the failure to observe purine synthesis in mammals with the aid of isotopic nitrogen or in microorganisms by analysis of growth requirements with the generally positive findings of feeding and perfusion experiments. Since the mechanism involves a "formate" intermediate rather than transfer of the imidazole ring as a unit, the nitrogen is lost, and failure of the conversion to occur in the microorganisms studied is merely evidence of a species difference.

With respect to creatine and creatinine, their low level of radioactivity shows that in the rat there is only a nominal contribution to the synthesis of these compounds. The data thus fail to support the idea of a specific precursor relationship between histidine and creatine-creatinine so far as position 2 is concerned. It is likely that the capacity of histidine to

increase the creatine output of rats subsisting on a diet deficient in protein (*4*) is due to the increased supply of ammonia and methyl groups available to the animal after receiving the histidine supplement, since many other amino acids, as well as ammonium citrate, also produce a rise. Interpretation of the behavior of protein-deficient rats is somewhat obscured by the fact that withdrawal of histidine alone from a normal diet causes a rise of creatine excretion (*6*). Since the withdrawal of histidine causes a severe loss of weight, the rise of creatine output may be a secondary result of general protein catabolism rather than a result of any specific connection between histidine and creatine synthesis. There remains the observation that incubation with histidine causes a rise of the creatine content of muscle brei (*12*). Under the conditions employed methyl donors do not produce the rise, and it is therefore possible that some portion of the histidine molecule which does not include position 2 might serve as an intermediate for creatine synthesis. Our study does not provide any information on this point.

Protoporphyrin and cholesterol were examined since they are known to be built up from small fragments, and it was of interest to determine whether histidine contributes to the synthesis of compounds of these classes. The absence of a significant isotope concentration in the porphyrin parallels the result obtained when formate is administered as such (*32*). Some isotope appears in the cholesterol, but the concentration is below that of carbon dioxide. Since examination of the body fat revealed no detectable radioactivity, the sterol question was not examined further.

Hepatoma group.—The hepatoma-bearing animals show no significant differences from the normal ones in their incorporation of isotope into the urinary purine end products or into cholesterol and protoporphyrin. Neither is there any difference between choline from normal and "precancerous" liver, but incorporation into tumor choline exhibits a lag. A second difference in the tumor choline is brought out in table 2, which gives the results of degradation experiments. Again there is no difference between normal and precancerous liver. In each case between 61 and 79 percent of the radioactivity is in the methyl groups. Choline from the hepatoma, however, contains isotope in the methyl groups only. If the incorporation of isotope into the ethanolamine moiety of choline arises by condensation of formate with glycine followed by decarboxylation of the serine so formed (*15*), the observed distribution of isotope in choline from the tumor indicates a pronounced impairment of one or both of these reactions in the *p*-dimethylaminoazobenzene hepatoma.

TABLE 2.—*Radioactivity of trimethylamine from choline degradation* [1]

	Normal liver			Precancerous liver		Hepatoma	
	12 hrs.	24 hrs.	72 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.
Trimethylamine	27. 0	37. 3	33. 2	28. 4	30. 8	8. 58	19. 2
Specific activity $(CH_3)_3N$ / Specific activity choline	. 611	. 789	. 664	. 638	. 692	. 991	1. 01

[1] Units as in table 1.

The differences in the urea values will be discussed in another paper.

SUMMARY

Carbon atom 2 of histidine is utilized in the biologic synthesis of purines and methyl groups by the rat. It is not used directly for the formation of creatine or creatinine. The relative contribution to purine synthesis is much greater than to methyl group synthesis. There is no significant contribution to cholesterol, body fat, or protoporphyrin. The distribution pattern indicates the participation of a "formate" intermediate.

The absence of isotope in the ethanolamine moiety of choline isolated from the *p*-dimethylaminoazobenzene hepatoma indicates that this tumor has little ability to carry out the sequence of condensing formate with glycine and decarboxylating serine.

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